

EXPRESS MAIL CERTIFICATE

Date December 28, 2001 Label No. ET 733483659 US  
I hereby certify that, on the date indicated above I deposited this paper  
or fee with the U.S. Postal Service and that it was addressed for delivery  
to the Assistant Commissioner for Patents, Washington, DC 20231  
by "Express Mail Post Office to Addressee" service.

464-1045- US

Julie Bowker  
Name (Print)

[Signature]  
Signature

5

10

**MULTIANALYTE MOLECULAR ANALYSIS USING APPLICATION-  
SPECIFIC RANDOM PARTICLE ARRAYS**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

15

This application is a continuation-in-part of International Application Serial No. PCT/US01/20179, filed June 21, 2001 which claims priority for U.S. Provisional Application Serial No. 60/213,106 filed June 21, 2000. All the above-referenced applications are expressly incorporated herein by reference

20

**FIELD OF THE INVENTION**

The present invention generally relates to the field of materials science and analytical chemistry.

25

The present invention discloses a process for the production of libraries of encoded magnetic particles, and the formation of planar assemblies inclusive of such particles. The present invention also discloses a platform for the implementation of multistep bioanalytical assay protocols permitting the integration of sample preparation steps with the simultaneous analysis of binding interactions between multiple types of analytes and binding agents.

30

## **BACKGROUND OF THE INVENTION**

Many bioanalytical procedures such as affinity purification and many biochemical assays such as immunoassays and DNA hybridization assays require the separation of specific molecules or constituents from a complex mixture. In the context of molecular and cell biology, magnetizable polymeric particles ("beads") have been widely used for this aspect of sample preparation. For example, magnetic beads displaying a short oligo-dT capture probe serve to extract messenger RNA (mRNA) molecules from a cell lysate. Following addition of particles to the lysate, molecules are captured by hybridization of their poly-A tails to the capture probe, trapped in a magnetic field gradient generated by a permanent magnet, retained by the applied magnetic field during the exchange of the lysate for fresh buffer and released into suspension by removal of the magnetic field ("Biomagnetic Techniques in Molecular Biology," Technical Handbook, 3<sup>rd</sup> Edition, DYNAL, 1998). In a similar manner, magnetic beads displaying antibodies directed against specific cell surface antigens serve to selectively extract cells of the desired type from a given suspension ("Cell Separation and Protein Purification", Technical Handbook, 2<sup>nd</sup> edition, DYNAL, 1997). A recent example describes a method of magnetic cell separation describes the use of magnetic particles in conjunction with optical imaging of trapped cells (A.G.J. Tibbe et al. "Optical tracking and detection of immunomagnetically selected and aligned cells" Nature Biotech., 17, 1999, 1210-1213).

The integration of assay steps, a principal objective motivating the introduction of clinical analyzers and other examples of laboratory automation, in today's state of the art relies upon a 96-well (or related) microwell format of multiple discrete reaction wells to accommodate standard robotic liquid handling ("pipetting") and reading of assay signals from individual wells by plate readers. Commercial robotic pipetting systems have been recently introduced to automate sample preparation based on the use of magnetic beads for separation. However, the integration of sample processing and a highly parallel array format of analysis by way of microfluidic operations, highly desirable in connection with the

miniaturization of biochemical and analytical assay procedures, has not been described to date.

The imprinting of multiple binding agents such as antibodies and oligonucleotides on planar substrates in the form of spots or stripes facilitates the simultaneous monitoring of multiple analytes such as antigens and DNA in parallel (“multiplexed”) binding assays. The miniaturization of this array format for increasing assay throughput and studying binding kinetics are described (R. Ekins, F. W. Chu, *Clin. Chem.* 37, 955-967 (1991); E. M. Southern, U. Maskos, J. K. Elder, *Genomics* 13, 1008-1017 (1992)). In recent years, this approach has attracted substantial interest particularly in connection with performing extensive genetic analysis (G. Ramsay, *Nat. Biotechnol.* 16, 40-44 (1998); P. Brown, D. Botstein, *Nat. Genet.* 21, 33-37 (1999); D. Duggan, M. Bittner, Y. Chen, P. Meltzer, J. M. Trent, *Nat. Genet.* 21, 10-14 (1999); R. Lipshutz, S. P. A. Fodor, T. R. Gingeras, D. J. Lockhart, *Nat. Genet.* 21, 20-24 (1999)).

The principal techniques of array fabrication reported to date include: refinements of the original “spotting” in the form of pin transfer or ink jet printing of small aliquots of probe solution onto various substrates (V. G. Cheung, et al., *Nat. Genet.* 21, 15-19 (1999)); sequential electrophoretic deposition of binding agents in individually electrically addressable substrate regions (J. Cheng, et al., *Nat. Biotechnol.* , 541-546 (1998)), and methods facilitating spatially resolved in-situ synthesis of oligonucleotides (U. Maskos, E. M. Southern, *Nucleic Acids Res.* 20, 1679-1684 (1992); S. P. A. Fodor, et al., *Science* 251, 767-773 (1991)) or copolymerization of oligonucleotides (A. V. Vasiliskov, et al., *BioTechniques* 27, 592-606 (1999)). These techniques produce spatially encoded arrays in which the position within the array indicates the chemical identity of any constituent probe (BioTechniques 27, 592-606 (1999)). All of these techniques of the prior invention require that array formation be completed prior to initiation of the assay of interest. Therefore, none of the techniques of array formation of the prior art permit the real-time formation of arrays subsequent to completion of the binding interaction of interest.

Monodisperse magnetic particles confined to planar substrates or interfaces, and exposed to a uniform magnetic field oriented normal to the plane of the interface, form a variety of ordered two-dimensional structures (W. Wen, L. Zhang and P. Sheng "Planar Magnetic Colloidal Crystals" Phys. Rev. Lett., 85, (25), 5464-5466, 2000; M. Golosovksy, Y. Saado, and D. Davidov "Self-assembly of floating magnetic particles into ordered structures: A promising route for the fabrication of tunable photonic band gap materials" Appl. Phys. Lett., 75, (26), 4186-4170, (1999); K. Zhan, R. Lenke, and G. Maret "Two-stage melting of paramagnetic colloidal crystals in two dimensions" Phys. Rev. Letter., 82, (13), 2721-2724, 1999).

10

Many techniques have been suggested for the synthesis of these particles. These techniques attempt to endow the magnetic particles with certain properties that make them desirable for certain applications. These techniques can be grouped into two categories, the first category relating to synthesis of a magnetic core and the second category relates to the synthesis of a magnetic shell.

15

Patents that may be considered of interest in the first category include:

U.S. Patent No. 4,358,388 to Daniel et al and U.S. Patent No. 5,356,713 to Charmot et al. disclose a process which utilizes a suspension polymerization approach.

One drawback of the process is the difficulty in controlling the mono-dispersity of the resulting magnetic Latex, and the process does not appear well suited for the generation of fluorescent magnetic particles

U.S. Patent Nos. 4,654,267 to Ugelstead et al discloses a nitration method which produces particles with a para-magnetic core. Following magnetization, the particles are coated with functional polymers to provide a reactive shell to produce super-paramagnetic particles of controlled morphology, polydispersity, pore size distribution, magnetic loading and surface chemistry. The encoding of such particles has not been described.

U.S. Patent No. 4,873,102 to Chang et al discloses a process of forming magnetic polymer particles containing crystals of magnetite uniformly throughout the pores. The particles can only be used under hydrophilic conditions.

30

U.S. Patent No. 5,356,713 to Charmot et al discloses magnetizable composite microspheres which are useful in biological applications but are limited by their size distribution to other applications.

U.S. Patent No. 5,512,439 to Hornes et al discloses monodisperse, super-magnetic particles carrying a plurality of molecules of an oligonucleotide which may be used for single stranded nucleic acids. The oligonucleotides may be covalently attached or affinity bonded.

U.S. Patent No. 5,698,271 to Liberti discloses a method for the manufacture of magnetically responsive particles. The particles have applications in a variety of preparative and diagnostic techniques.

U.S. Patent No. 5,866,099 to Owen et al discloses a magnetic-polymer particle useful in immunoassay techniques and biological/medical applications. The particle is produced by co-precipitation of transition metals in the presence of a polymer having available coordination sites.

15

Patents that may be considered of interest in the second category include:

U.S. Patent No. 5,736,349 to Sasaki et al discloses a magnetic particle for an immunoassay method which comprises a core and a coating layer. An antigen or antibody is bound onto the surface of the coating layer.

U.S. Patent No. 5,648,124 to Sutor et al discloses a process for the production of magnetic particles by hetero-coagulation utilizing oppositely charged core particles and magnetite particles. The dispersed magnetite may be a coated microparticle which can be further coated with one or more outer polymeric coatings.

U.S. Patent Nos. 6,013,531, 5,283,079 and 5,091,206 to Wang et al disclose a process for producing magnetically responsive polymer particles. The particles comprise a polymeric core particles coated evenly with a layer of polymer containing magnetically responsive metal oxide. The surface of these magnetically responsive polymer particles can be coated further with a layer of functionalized polymer. These magnetically responsive polymer particles can be used for passive or covalent coupling of biological material and used as solid phase for various types of immunoassays.

Several methods have been described for the synthesis of stained magnetic particles. Patents that may be considered of interest include U.S. Patent No. 5,395,688 to Wang which discloses a process for producing magnetically responsive fluorescent polymer particles composed of a fluorescent polymer core particle that is evenly coated with a layer of magnetically responsive metal oxide. The method utilizes preformed fluorescent polymeric core particles which are mixed with an emulsion of styrene and magnetic metal oxide in water and polymerized. A two-step reactive process such as this suffers from the drawback of possible inhibition of polymerization by the fluorescent dye or conversely bleaching of the fluorescence by the shell polymerization process. The use of such magnetic particles containing fluorescent tags for the calibration of certain solid phase assays has been described in U.S. Patent No. 6,013,531. Following completion of this step, the particles are coated with functional polymers to provide a reactive shell.

The creation of core-shell particles from dispersed colloidal matter can be accomplished by a multistep (layer-by-layer) strategy. The process involves step-wise adsorption of charged polymers or nanoparticles and oppositely charged polyelectrolytes onto colloidal particles, exploiting primarily electrostatic interactions for layer buildup. (Caruso et al "Magnetic Core-Shell Particles: Preparation of Magnetite Multilayers on Polymer Latex Microspheres" Adv. Mater. 1999, 11, 950-953) A shell applied by electrostatic physisorption is not desirable for bioanalytical assays because it is chemically unstable under changes of assay conditions, particularly salt concentration, and promotes non-specific adsorption and denaturation of charged biomolecules; the particles described in this prior art reference are unsuitable in connection with the assay formats contemplated herein.

While the foregoing references disclose the use of magnetic particles, none of the prior art particles appear to possess the properties that are necessary to meet the criteria which are necessary for the successful performance of the assays described herein including a preferred size range, substantial monodispersity, chemical functionalization and

synthetic flexibility, the latter permitting the rapid construction of libraries of encoded magnetic particles that can be functionalized on demand, the chemical diversity represented in these libraries greater than 2. In addition, magnetic particles must meet certain standards of quality to permit the reproducible assembly of customized arrays to ensure consistent  
5 performance in quantitative assays.

### **SUMMARY OF THE INVENTION**

The present invention provides methods, compositions and processes relating to the  
10 implementation of bioanalytical assay procedures for multiplexed molecular interaction analysis. The processes of the invention employ a multiplicity of encoded magnetic particles, distinguishable on the basis of their encoding. Such particles display one or more binding agents which are capable of interacting with one or more specific analytes upon coming into contact such particles.

15

The present invention provides a process for the preparation of libraries of particles which are a composite of an encoded particle and a multitude of magnetic nanoparticles ("composite particles"). Such composite particles can be functionalized to be able to be utilized in a particular assay. Examples of such functionalization include the inclusion of  
20 binding agents such as oligonucleotides, DNA, peptides or proteins. Such composite particles include an optically differentiable code, such code selected to reflect the nature of the displayed binding agent, such that the composite particles and their associated binding agents are distinguishable by real time, in-situ inspection. Custom bead arrays can be fabricated on demand using such libraries. Such arrays are useful in bioassays, including  
25 assays involving multiplexed molecular interaction analysis.

The bioanalytical assay platform disclosed herein utilizes libraries of composite particles to integrate principal assay steps including sample capture and preparation, processing and analysis. In a preferred embodiment, analysis is performed in a highly

parallel bead array format in which arrays are assembled in real time following completion of sample preparation and processing steps.

### **BRIEF DESCRIPTION OF DRAWINGS**

5

Other objects, features and advantages of the invention discussed in the above brief explanation will be more clearly understood when taken together with the following detailed description of an embodiment which will be understood as being illustrative only, and the accompanying drawings reflecting aspects of that embodiment, in which:

10

Figure 1 is an illustration of process flow including the production of random encoded bead arrays and their use in multiplexed assays.

Figure 2 is an illustration of the functionalization of beads.

15

Figure 3 is an illustration of steps in chip design and wafer-scale production.

Figure 4 is an illustration of on-demand assembly of random encoded arrays.

Figure 5 is an illustration of palmtop microlab.

Figure 6 is a schematic illustration of assay and decoding images used in the random encoded array detection process of the invention.

20

Figure 7 is a flow chart summarizing algorithms and steps in the analysis of images.

Figure 8 is an illustration of steps in the decomposition of assay images according to bead type by application of the image analysis algorithm summarized in Figure 7.

25

Figure 9 is an illustration of optically programmable array assembly of random encoded arrays.

Figure 10 is an illustration of an array composed of random encoded subarrays.

Figure 11 is an illustration of stations in an automated chip-scale bead array manufacturing and quality control process.

30



Figure 12 is an illustration of quantitative binding curves for two cytokines.

Figure 13 is an illustration of array design for polymorphism analysis.

Figure 14 is a fluorescence micrograph of assay and decoding images recorded from one subarray shown in Figure 13 in the course of polymorphism analysis

5                Figure 15 is an illustration of assay results in the form of intensity histograms obtained from the analysis of assay images such as the one illustrated in Figure 14.

Figure 16 is an illustration of design of a “looped probe” for hybridization assays.

10              Figures 17A and 17B are fluorescence micrographs of assay and decoding images recorded in the course of the analysis of multiple cytokines.

Figures 18A and 18B are illustrations of numerical simulations of cross-correlations in receptor-ligand systems with multiple competing receptor-ligand interactions.

15              Figure 19 is an illustration of numerical simulations of receptor-ligand association and disassociation kinetics.

Figure 20 is an illustration of integrated sample capture using magnetic capture beads and array-based detection using the random encoded array detection process of the invention.

20              Figure 21 is an illustration of multi-step assays using composite particles to integrate gene-specific capture, on-bead reverse transcription and post-assay array assembly.

Figure 22 is an illustration of multi-step assay sequence having applicability to a wide range of biochemical assays.

25              Figure 23(i) is a histogram showing four different types of single optically particles and Figure 23(ii) is a dot plot of eight dual encoded particles.

Figure 24 is an illustration of the variation of magnetic loading.

Figure 25 is an illustration the composite particles of the invention exposed to a magnetic field.

Figure 26 is a schematic illustration of the experimental assembly for application of a magnetic field.

Figure 27(i) is a schematic illustration of the 2D structure of the bead assembly before application of a magnetic field. Figure 27(ii) is a schematic illustration of the 2D structure of the bead assembly after application of a magnetic field of about 100 Gauss. Figure 27(iii) is a schematic illustration of a close-up of 2D assembly in Figure 27(ii). Figure 27(iv) is a schematic illustration of the 2D arrangement for a higher (2x) bead concentration under a magnetic field of about 20 Gauss.

Figure 28 shows results of a streptavidin-biotin binding assay using the encoded and magnetic particles of the invention.

Figure 29 shows results of a hybridization assay using the encoded and magnetic particles of the invention.

#### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

15

The fabrication of application-specific bead arrays may involve multiple processes in a multi-step sequence which may be automated using existing liquid handling technology and laboratory automation. The process described herein and referred to as random encoded array detection (“READ”) includes the fabrication of random encoded arrays as well as the use of such arrays in bioassays, including assays involving multiplexed molecular interaction analysis, including but not limited to the interaction of analyte and binding agent molecules, as exemplified by DNA and protein analysis. Random encoded arrays, as described herein, as well as in U.S. Patent No. 6,251,691 overcome many of the shortcomings associated with processes employing multi-step sequences.

25

As used herein, the terms “analyte” and “binding agent” refer to molecules involved in binding interactions. By way of example, analyte and binding agent may include DNA or RNA fragments (e.g., oligonucleotide), aptamers, peptides, and proteins, antigens and small organic molecules. In a particular assay, binding of these fragments to their complementary

sequences (hybridization) is analyzed. In another particular assay, binding interactions between ligands and receptors are analyzed.

As used herein, the term “particles” refers to colloidal particles and beads. The term “particle” is also used in connection with the encoded particles and magnetic particles of the invention.

As used herein, the term “magnetic particle” refers to a particle possessing a permanent or induced dipole moment.

10

Figure 1 provides a schematic overview of the functional components and process flow by which custom bead arrays may be prepared and used in performing multiplexed biomolecular analysis according to the present invention. The array is prepared by employing separate batch processes to produce application-specific substrates (e.g., chip at the wafer scale), to produce beads that are encoded and functionalized (e.g., at the scale of  $\sim 10^8$  beads/100  $\mu$ l of suspension) or to produce beads that are encoded, magnetic and functionalized. The beads subjected to respective quality control (QC) steps prior to array assembly, such as the determination of morphological and electrical characteristics. In addition, actual assays are performed on beads in suspension, before they are introduced to the substrate, to optimize assay conditions, generally with the objective to maximize assay sensitivity and specificity and to minimize bead-to-bead variations. For substrates, QC steps may include optical inspection, ellipsometry and electrical transport measurements.

Once the chemically encoded and biologically functionalized beads are combined with the substrate (e.g., chip), the process described in U.S. Patent No. 6,251,691 (“LEAPS) in conjunction with the process described in PCT/US97/08159 may be used for rapid assembly of dense arrays on a designated area on the substrate within the same fluidic phase, avoiding problems contributing to spot-to-spot as well as chip-to-chip variability without the need for retooling or process redesign. Furthermore, the bead array format permits chip-independent characterization of beads as well as optimization of assay

30

conditions. In addition, multiple bead arrays can be formed simultaneously in discrete fluid compartments maintained on the same chip. Once formed, these multiple bead arrays may be used for concurrent processing of multiple samples. The integration of LEAPS with microfluidics produces a self-contained, miniaturized, optically programmable platform for parallel protein and DNA analysis. U.S. Patent No. 6,251,691 and PCT/US97/08159 are incorporated herein by reference in their entirety.

In certain embodiments of the present invention, chemical encoding may be accomplished by staining beads with sets of optically distinguishable tags, such as those containing one or more fluorophore dyes spectrally distinguishable by excitation wavelength, emission wavelength, excited-state lifetime or emission intensity. The optically distinguishable tags may be used to stain beads in specified ratios, as disclosed, for example, in Fulwyler, U.S. Patent No. 4,717,655. Staining may also be accomplished by swelling of particles in accordance with methods known to those skilled in the art, (Molday, Dreyer, Rembaum & Yen, J. Mol Biol 64, 75-88 (1975); L. Bangs, "Uniform Latex Particles, Seragen Diagnostics, 1984]. For example, up to twelve types of beads were encoded by swelling and bulk staining with two colors, each individually in four intensity levels, and mixed in four nominal molar ratios. Combinatorial color codes for exterior and interior surfaces is disclosed in International Application No. PCT/US/98/10719, which is incorporated herein by reference in its entirety.

Beads are functionalized by binding agent molecules attached thereto, the molecule including DNA (oligonucleotides) or RNA fragments, peptides or proteins, aptamers and small organic molecules in accordance with processes known in the art, e.g., with one of several coupling reactions of the known art (G. T. Hermanson, Bioconjugate Techniques (Academic Press, 1996); L. Illum, P. D. E. Jones, Methods in Enzymology 112, 67-84 (1985). In certain embodiments of the invention, the functionalized beads have binding agent molecules (e.g., DNA, RNA or protein) covalently bound to the beads. Beads may be stored in a buffered bulk suspension until needed. Functionalization typically requires one-step or two-step reactions which may be performed in parallel using standard liquid

handling robotics and a 96-well format to covalently attach any of a number of desirable functionalities to designated beads, as illustrated in Figure 2. In a preferred embodiment, beads of core-shell architecture will be used, the shell composed in the form of a thin polymeric blocking layer whose preferred composition is selected; and functionalization  
5 performed in accordance with the targeted assay application, as known in the art. Samples may be drawn for automated QC measurements. Each batch of beads provides material for hundreds of thousands of chips so that chip-to-chip variations are minimized.

Substrates (e.g., chips) used in the present invention may be patterned in  
10 accordance with the interfacial patterning methods of LEAPS, by for example, patterned growth of oxide or other dielectric materials to create a desired configuration of impedance gradients in the presence of an applied AC electric field. Patterns may be designed so as to produce a desired configuration of AC field-induced fluid flow and corresponding particle transport. Substrates may be patterned on a wafer scale by invoking semiconductor  
15 processing technology, as illustrated in Figure 3. In addition, substrates may be compartmentalized by depositing a thin film of a UV-patternable, optically transparent polymer to affix to the substrate a desired layout of fluidic conduits and compartments to confine fluid in one or several discrete compartments, thereby accommodating multiple samples on a given substrate.

20

In certain embodiments of the invention, the bead array is prepared by providing a first planar electrode that is substantially parallel to a second planar electrode ("sandwich" configuration) with the two electrodes being separated by a gap and containing an electrolyte solution. The surface or the interior of the second planar electrode is patterned  
25 with the interfacial patterning method. Encoded and functionalized beads are introduced into the gap. When an AC voltage is applied to the gap, the beads form a random encoded array on the second electrode (e.g., "chip"). And, also using LEAPS, an array of beads may be formed on a light-sensitive electrode ("chip"). Preferably, the sandwich configuration described above is also used with a planar light sensitive electrode and  
30 another planar electrode. Once again, the two electrodes are separated by the a gap and

contain an electrolyte solution. The functionalized and encoded beads are introduced into the gap. Upon application of an AC voltage in combination with a light, the beads form an array on the light-sensitive electrode.

5           In certain embodiments, the application-specific bead arrays useful in the present invention may be produced by picking aliquots of designated encoded beads from individual reservoirs in accordance with the specified array composition and “pooled”; aliquots of pooled suspension are dispensed onto selected substrate (e.g., chips) in a manner preventing the initial fusion of aliquots. Aliquots form a multiplicity of planar random subarrays of encoded beads,  
10 each subarray representing beads drawn from a distinct pool and the physical array layout uniquely corresponding to the identity of aliquots drawn from pooled bead populations.

Planar arrays or assemblies of encoded beads on a substrate which are chemically or physically encoded may be used. To this, spatial encoding may also be added to increase  
15 the number of assays that may be conducted. Spatial encoding, for example, can be accomplished within a single fluid phase in the course of array assembly using LEAPS to assemble planar bead arrays in any desired configuration in response to alternating electric fields and/or in accordance with patterns of light projected onto the substrate. LEAPS creates lateral gradients in the impedance of the interface between silicon chip and solution  
20 to modulate the electrohydrodynamic forces that mediate array assembly. Electrical requirements are modest: low AC voltages of typically less than  $10V_{pp}$  are applied across a fluid gap of typically  $100\text{ }\mu\text{m}$  between two planar electrodes. This assembly process is rapid and it is optically programmable: arrays containing thousands of beads are formed within seconds under electric field. The formation of multiple subarrays, can also occur in  
25 multiple fluid phases maintained on a compartmentalized chip surface.

The multiplexed assays of the present invention may also be performed using encoded beads that are assembled, but not in an array, on the substrate surface. For example, by spotting bead suspensions into multiple regions of the substrate and allowing  
30 beads to settle under gravity, assemblies of beads can be formed on the substrate. In

contrast to the bead arrays formed by LEAPS, these assemblies generally assume low-density, disorder configurations. However, the combination of spatial and color encoding attained by spotting mixtures of chemically encoded beads into a multiplicity of discrete positions on the substrate still provides a degree of multiplexing that is sufficient for  
 5 certain biological assays.

Binding interaction between the binding agent on those beads and an analyte may be performed either before or after the encoded array is assembled on the substrate. For example, the bead array may be formed after the assay, subsequent to which an assay image  
 10 and a decoding image may be taken of the array. Alternatively, the beads may be assembled in an array and immobilized by physical or chemical means to produce random encoded arrays, e.g., with the appearance of the array shown in Figure 10. The arrays may be immobilized, for example, by application of a DC voltage to produce random encoded arrays with the appearance of the array shown in Figure 10. The DC voltage, set to  
 15 typically 5-7 V (for beads in the range of 2-6  $\mu\text{m}$  and for a gap size of 100-150  $\mu\text{m}$ ) and applied for < 30s in “reverse bias” configuration so that an n-doped silicon substrate would form the anode, causes the array to be compressed to an extent facilitating contact between adjacent beads within the array and simultaneously causes beads to be moved toward the region of high electric field in immediate proximity of the electrode surface. Once in  
 20 sufficiently close proximity, beads are anchored by van der Waals forces mediating physical adsorption. This adsorption process is facilitated by providing on the bead surface a population of “tethers” extending from the bead surface; polylysine and streptavidin have been used for this purpose.

25 In certain embodiments, the particle arrays may be immobilized by chemical means, for example, by forming a composite gel-particle film. In one example for forming such gel-composite particle films, a suspension of microparticles is provided which also contains all ingredients for subsequent in-situ gel formation, namely monomer, crosslinker and initiator. The particles are assembled into a planar assembly on a substrate by application of  
 30 LEAPS, e.g., AC voltages of 1-20  $V_{p-p}$  in a frequency range from 100's of hertz to several

kilohertz are applied between the electrodes across the fluid gap. Following array assembly, and in the presence of the applied AC voltage, polymerization of the fluid phase is triggered by thermally heating the cell  $\sim 40\text{-}45^{\circ}\text{C}$  using an IR lamp or photometrically using a mercury lamp source, to effectively entrap the particle array within a gel. Gels may be composed of a mixture of acrylamide and bisacrylamide of varying monomer concentrations from 20% to 5% (acrylamide : bisacrylamide = 37.5 : 1, molar ratio), or any other low viscosity water soluble monomer or monomer mixture may be used as well. Chemically immobilized functionalized microparticle arrays prepared by this process may be used for a variety of bioassays, e.g., ligand receptor binding assays.

In one example, thermal hydrogels are formed using azodiisobutyramidine dihydrochloride as a thermal initiator at a low concentration ensuring that the overall ionic strength of the polymerization mixture falls in the range of  $\sim 0.1\text{mM}$  to  $1.0\text{mM}$ . The initiator used for the UV polymerization is Irgacure 2959<sup>®</sup> (2-Hydroxy-4'-hydroxyethoxy-2-methylpropiophenone, Ciba Geigy, Tarrytown, NY). The initiator is added to the monomer to give a 1.5 % by weight solution.

In certain embodiments, the particle arrays may be immobilized by mechanical means. For example, an array of microwells may be produced by standard semiconductor processing methods in the low impedance regions of the silicon substrate. The particle arrays may be formed using such structures by, for example, utilizing LEAPS mediated hydrodynamic and ponderomotive forces are utilized to transport and accumulate particles on the hole arrays. The AC field is then turned off and particles are trapped into microwells, and are therefor, mechanically confined. Excess beads are removed leaving behind a geometrically ordered random bead array on the substrate surface.

When the bead array is immobilized before the assay, the array functions as a two-dimensional affinity matrix which displays receptors or binding agents (e.g., oligonucleotides, cDNA, aptamers, antibodies or other proteins) to capture analytes or ligands (DNA, proteins or other small cognate ligands) from a solution that is brought in



contact with the array. The bead array platform may be used to perform multiplexed molecular analysis, such as, e.g., genotyping, gene expression profiling, profiling of circulation protein levels and multiplexed kinetic studies.

5           Substrates (e.g., chips) can be placed in one or more enclosed compartments, permitting samples and reagents to be transported in and out of the compartments through fluidic interconnection. On-chip immunoassays for cytokines, e.g., interleukin (IL-6) may be performed in this format. Serum sample and fluorescent labeled secondary antibodies are introduced to the reaction chamber sequentially and allowed to react with beads  
10 immobilized on the chip. Figure 5 illustrates a design of a reaction chamber which may be used in the multiplexed assays according to the present invention. Reactions can also be performed in an open compartment format similar to microtiter plates. Reagents may be pipetted on top of the chip by robotic liquid handling equipment, and multiple samples may be processed simultaneously. Such a format accommodates standard sample processing and  
15 liquid handling for existing microtiter plate format and integrates sample processing and array detection.

In certain embodiments, the presence of the analyte-binding agent interactions are associated with changes in the optical signatures of beads involved in the interactions and  
20 these optical changes detected and analyzed. The identities of the binding agents involved in the interactions are determined by detecting the chemically or physically distinguishable characteristic associated with those beads. Preferably, chemically distinguishable characteristics include chemical molecules including fluorphore dyes, chromophores and other chemical molecules that are used for purposes of detection in binding assays.

25

The detection of the chemically or physically distinguishable characteristic and the detection of optical signature changes associated with the binding interactions may be performed while the particles are assembled in a planar array on a substrate, for example, by taking an assay and a decoding image of the array and comparing the two, for example,  
30 a comparison of the assay and the decoding image comprises the use of optical microscopy

apparatus including an imaging detector and computerized image capture and analysis apparatus. The decoding image may be taken to determine the chemically and/or physically distinguishable characteristic that uniquely identifies the binding agent displayed on the bead surface, for example, by determining the identity of the binding agents on each particle in the array by the distinguishable characteristic. The assay image of the array is taken to detect the optical signature of the binding agent and the analyte complex. In certain embodiments, fluorescent tags (fluorophore dyes) may be attached to the analytes such that when the analytes are bound to the beads, the fluorescent intensities change, thus providing changes in the optical signatures of the beads. In certain embodiments, the decoding image is taken after the beads are assembled in an array and immobilized and before taking the assay image, preferably before contacting the binding agents on the beads with an analyte. In certain other examples, the binding interactions occur while the beads are in solution, and assembled into an array afterwards and the decoding and assay images are obtained.

The identity of the binding agent of the binding agent-analyte complex is carried out by comparing the decoding image with the assay image.

In preferred embodiments, images analysis algorithms that are useful in analyzing the data obtained from the decoding and the assay images. These algorithm may be used to obtain quantitative data for each bead within an array. As summarized in Figure 7, the analysis software automatically locates bead centers using a bright-field image of the array as a template, groups beads according to type, assigns quantitative intensities to individual beads, rejects "blemishes" such as those produced by "matrix" materials of irregular shape in serum samples, analyzes background intensity statistics and evaluates the background-corrected mean intensities for all bead types along with the corresponding variances.

The methods of the present invention may be used for determining the association and the dissociation constants e.g., by introducing the analyte in a time-dependent manner

and analyzing the binding as a function of time, or by washing away the bound analyte in a time-dependent manner and also analyzing the binding as a function of time.

The methods of the present invention may be used for determining the affinity  
5 constants of analyte-binding agent interactions, for determining the number of analyte-binding agent complexes formed

The present invention also provides methods for determining the concentration of an analyte in a biological sample.

10

The methods of the present invention may also be used to determining elements of a co-affinity matrix of a given analyte against a panel of binding agents. In one example, the extent of the interaction between the analyte and the binding agents in a panel in competitive, multiconstituent equilibrium reaction may be determined. Determination of  
15 co-affinity constants provides useful applications, as described below.

The successful rate of transplantation for several types of organs directly relates to compatibility of Human Leukocyte Antigen (HLA) between donor and recipient. Serological testing of the recipients for the Panel Reactive Antibodies (PRA) is one of the  
20 crucial steps to avoid possible rejections. Cross-reaction in PRA testing is a very common phenomenon due to similarity of some HLA antigen structures and the nature of development of these antibodies. In fact, HLA antigens can be organized into groups based on apparent serological cross-reactivity between the groups. These groups are termed Cross-Reactive-Groups (CREGs). In current clinical setting, antibodies from a patient are  
25 tested against different antigens in individual reactions. Although a reactive pattern of the antibodies can be generated combining the results from different reactions, the competitive nature of interactions between different antibodies and antigens is not reflected in such a pattern. In other cases, several antigens are mixed together for a binding assay. Lack of identification of each antigen in the system prevents generation of a binding profile. The  
30 result is only the averaged signal from several antigens. In the bead array system, a panel of

different antigens is presented to the antibody analytes in a competitive binding environment, and each antigen can be identified through its association with different types of beads. Thus, binding intensity on each antigen in the competitive reactions can be extracted in a single assay. This co-affinity matrix system will provide binding profiles for the CREGs and greatly advance the understanding of the nature of the reaction and improve the accuracy for the related clinical decisions. For example, a N-antibody and M-antigen system provides a matrix of N x M of possible reactions. It is possible to determine  $K_{nm}$ , the affinity constant governing the interaction between the nth antibody against the mth antigen, where  $m = 1, 2, \dots, M$ , and  $n = 1, 2, \dots, N$ . For applications where absolute co-affinity constants are not needed, binding profile will be generated for various antibodies in accordance with the methods of the present invention and results from a patient sample can be matched to these profiles or combination of these profiles.

Co-affinity matrix may also be used to characterize the analyte. For example, combination of the coefficients of the co-affinity matrix and known concentrations of analyte and binding agents participating in the formation of analyte-binding agent complexes serves to define a competitive binding interaction descriptor, e.g., The molecular interaction parameter,

$$P_n(R_m) = \frac{K_{mn}[L_n]}{\sum_j K_{mj}[L_j]}$$

provides a characterization of the molecular interaction between a binding agent,  $R_m$ , and an analyte,  $L_n$ , in the presence of analytes  $\{L_j; 1 \leq j \leq N\}$ , all of which exhibit a finite affinity,  $K_{mj}$ , for that binding agent. That is,  $P_n, 0 \leq P_n \leq 1$ , represents a normalized specificity of binding agent  $R_m$  for analyte  $L_n$  in a multiconstituent competitive reaction and

serves as a robust characterization of that binding agent based on co-affinities displayed in a multiconstituent competitive reaction. See also P.H. von Hippel et al., Proc. Natl. Acad. Sci. USA 83, 1603 (1986), incorporated herein by reference.

5           The pattern of binding interaction of a analyte against a panel of binding agents may be used to characterize the analyte and compare it with other molecules. In addition, by generating the co-affinity matrix of a analyte using a reference panel of binding agents, such affinity may be used to determine if a sample later introduced to the panel of binding agents contains an impurity by observing the deviation in the binding pattern.

10

          The present invention also provides use of superparamagnetic particles ("magnetic particles") as described in U.S. Patent No. 5, 759,820 and European Patent No. 83901406.5 (Sintef), which may then be used in integrated the sample preparation step with the assay step involving encoded bead arrays. Both of these references are incorporated  
15   herein by reference.

          Superparamagnetic particles may be encoded with a chemically or physically distinguishable characteristic (e.g., fluorescent tag) and used performing bioassays of the present invention. In certain embodiments, the particles are assembled using LEAPS, as  
20   with non-magnetic encoded beads. The encoded nanoparticles can be also be used in array generation, and assayed. The present invention also includes the formation of a planar array of encoded and functionalized superparamagnetic particles on a substrate by application of magnetic field to said particles.

25           The invention provides a novel process for making color encoded magnetic beads, a simple and flexible one-step process to introduce into preformed polymeric microparticles a well controlled amount of magnetic nanoparticles, prepared in accordance with the procedure described below, along with well controlled quantities of one or more fluorescent dyes. In an embodiment of the present invention, the quantity of the magnetic  
30   nanoparticles. is controlled to produce magnetic particles that form an array on a substrate

upon application of magnetic field to said particles. This process involves swelling the polymer particles in an organic solvent containing dyes and magnetic nanoparticles and therefore applies to any polymer particle which can be subjected to standard swelling procedures such as those disclosed in the prior art of fluorescent staining of microparticles .

- 5 Unlike encoding methods in which the magnetic material and the fluorescent dyes are each located to different areas of the (core/shell) of the magnetic particle, uniform swelling of particles ensures the distribution of magnetic particles throughout the interior volume. This process also permits the quantitative control of the nanoparticle as well as dye content over a wide range, thereby permitting the tailoring of the particles' magnetic susceptibility as
- 10 well as fluorescence intensities. An additional method of the present invention to control the magnetic properties of the host particles, other than to control loading, is to tune the size of the magnetic nanoparticles by adjusting the water content of the micellar synthesis reaction (see below). Physical or chemical coupling of biomolecules possible on the particle surface utilizing preexisting functional groups. Leaching out of magnetic nanoparticles is
- 15 readily eliminated by growing a further polymeric shell on the particle.

- The invention also provides a novel process for the production of libraries of encoded magnetic particles, by a two-step process. The first step in the process involves the production of a sub-library of magnetic nanoparticles and the second step in the process
- 20 involves the production of a sub-library of encoded particles. Following sub-library formation, as more specifically described below, the members of each sub-library are endowed with a coupling site and also functionalized

- The sub-library of magnetic nanoparticles can be produced by combining a metal
- 25 oxide dispersion with a monomer solution and forming a polymer matrix of particles encapsulating particles of metal oxide. The metal oxide dispersion may include a metal oxide, used alone or in combination with a different metal oxide. The metal oxide may include without limitation, oxides of iron, manganese, cobalt, zinc, nickel and copper, or the like. The monomer solution may include a monomer, used alone or in combination
- 30 with a different monomer. The monomer may include without limitation, styrene, methyl-

methacrylate, acrylamide, ethylene-glycol acrylate, hydroxy-ethyl-methacrylate, vinyl-toluene, divinyl-benzene, or the like. Alternatively, a polymer solution can be used and may include a polymeric material such as cellulose, proteinaceous polymer, glass, agarose, gelatin, or the like. The magnetic nanoparticles may be on any size and shape, but are  
5 preferably, spherical, monodisperse and from about 0.01 to about 1 micron, preferably from about 0.05 to about 0.4 microns.

The sub-library of encoded particles can be formed by encapsulating an optical identifier in a polymeric material. The polymeric material may include one polymer, used  
10 alone or in combination. The polymers may include without limitation, polystyrene, polymethyl-methacrylate, poly-acrylamide, poly-ethylene-glycol, poly-hydroxy-ethyl-methacrylate, polyvinyl-toluene, poly-divinyl-benzene, brominated polystyrene, polyacrolein, polyethylene, polyurethane, polyvinyl alcohol, polyvinylchloride or combinations thereof, or the like. The optical identifier may include one or more dyes with  
15 emission wavelengths between 200 nm and 1200 nm. Preferred dyes include but are not limited to pyro-methane and coumarin dyes. More than one dyes with distinguishable emission spectra can be used simultaneously and are selected based on their emission spectra.

20 The encoded particles of the invention may be of any size and shape, but are preferably polymeric, spherical, monodisperse and from about 0.6 to about 100 microns, preferably from about 1.5 to about 10 microns.

Depending on the application of interest as may be illustrated from the embodiments  
25 and examples that follow, each sub-library of the invention can be customized. In one embodiment, such customization can be accomplished by endowing the magnetic nanoparticles with a coupling site or a functional site which can both be selected based on the application of interest. Such sites can be formed by attachment of a molecular group of interest to the outside surface of the magnetic particle. The coupling sites may be formed  
30 during the polymerization step and the functional sites may be formed at a later stage, prior

to formation of the composite particles as illustrated below. Molecular entities of applicability to the invention for the generation of a coupling site include without limitation any group such as carboxylic, ester, amine, aldehyde, alcohol, or halide, streptavidin, avidin, neutravidin, biotin or the like. Molecular entities of applicability to the invention for the generation of a functional site include without limitation streptavidin, avidin, neutravidin, biotin, protein A or the like. The pre-selection of a molecular entity to endow the magnetic nanoparticles of the invention with a particular functional site provides the ability to compile a certain class of sub-libraries which can be useful in the performance of certain assays. In an illustration of the embodiment of this invention, in order to perform an assay for detecting the presence of a nucleic acid of interest, one would provide the applicable functional site to enable the coupling of one or more oligonucleotide or cDNA. In another illustration of the embodiment of this invention, to perform an assay for detecting the presence of a protein of interest, one would provide a functional site to enable the coupling of one or more antigen or antibody which are specific to the detection or modulation of the protein of interest. Such functional coupling can be accomplished prior to or following forming the composite particles of the invention.

Generally, the foregoing functional and coupling groups can be attached to the outside surface of the magnetic nanoparticle by covalent attachment using known methods in the art. Following formation of the site of interest on the magnetic nanoparticles, the sub-library of magnetic nanoparticles can be brought in contact with the sub-library of encoded particles and the magnetic nanoparticles can be caused to attach to the encoded particles through the coupling site using known method in the art, while leaving the functional site unattached and available for the performance of assay of interest.

25

In another application of the invention, as illustrated in the following Examples, the metal oxide loading of the composite particles of the invention can be controlled and adjusted so as to induce a magnetic response of interest. The foregoing can be accomplished by controlling the number of magnetic nanoparticles which bind to the surface of the encoded particle.

30



In another application of the invention, as illustrated in the following Examples, the optical identifier in the encoded particles can be incorporated in specified ratios to accomplish the objectives of the application of interest. The control of the ratio of the optical identifier can be accomplished using known methods in the art such as for example  
 5 the method disclosed in U.S. Patent No. 4,717,655.

Following customization of the sub-library of magnetic nanoparticles, as illustrated in the following Examples, a library of composite particles can be formed by covalently attaching a multitude of functionalized magnetic nanoparticle to the outer surface of an  
 10 encoded particle, the properties of the particles being pre-selected depending on the assay to be performed. As exemplified herein, the chemical diversity represented in the library of the composite particles of the invention is greater than 2. In another embodiment, the composite particles of the invention may be produced during execution of the assay itself. The assay invokes the binding interaction between one functional group displayed on a  
 15 magnetic nanoparticle of the invention and a second molecular group displayed on the encoded particle of the invention to produce the composite particle of the invention. In the course of the assay, heterostructures composed of magnetic and non-magnetic particles can be formed as a result of the formation of a complex between functional and molecular groups following which the composite particles of the invention can be arranged into array  
 20 format for detection.

The composite particles of the invention can be manipulated on a substrate which may be patterned in accordance with the interfacial patterning methods disclosed in U.S. Patent No. 6,251,691. Following patterning, arrays of the composite particles of the  
 25 invention can be prepared utilizing the “sandwich” configuration.

The composite particles of the invention can also be manipulated and assembled into ordered arrays in response to magnetic fields. The presence of magnetic field gradients induces a force on the composite particles ( $\sim \frac{1}{2}(\chi V/\mu_0)\text{del}(B^2)$ ), where  $\chi$  the magnetic  
 30 susceptibility of a composite particle, V its volume, B magnitude of the magnetic field (flux

density)) the direction and magnitude of which depends on the exact nature of the magnetic field distribution and the strength of the magnetic field. A proper combination of an uniform axial field and localized field gradient generating structures can be used to capture and immobilize (reversibly) the composite particles of the invention in an array format. Such a capability of being able to form 2D arrays as well as immobilize such particles has particular applicability in connection with the performance of multi-step assays involving buffer exchange and multiple wash steps. A particularly useful structure for generating a uniform axial as well as tunable gradient in the vertical direction is a pair of electromagnetic coils such that their individual field directions oppose each other. Hence by adjusting the gap between the coils or the relative position of the sample with respect to the coils and by varying the individual currents in the coils, the force on the composite particles of the invention ( $\frac{1}{2}(\chi V/\mu_0) \text{ dB/dz}$ ) can be controlled.

As a function of increasing magnetic field strength, ordered planar assemblies of field-dependent number density (or equivalently, average inter-particle distance) and linear strings of beads oriented normal to the substrate can be formed.

In a preferred embodiment, planar structures are formed at or near a substrate by application of a uniform magnetic field oriented normal to the substrate plane. For typical values of magnetic susceptibility of composite particles with an average diameter of 3.2  $\mu\text{m}$ , prepared as described in Examples provided herein, a field producing a magnetic induction in the range of approximately 1,000 to 2,000 Gauss suffices to produce planar assemblies. Permanent magnets are available which, when placed in immediate proximity to the substrate, generate a field near the substrate surface of sufficient field strength to realize the desired planar configuration of the assembly. Requisite magnetic fields configurations also can be produced by an electromagnet, for example in solenoid or Helmholtz configuration known to the art; the substrate can be introduced into the magnet bore or can be placed in immediate proximity to the coil(s) outside of the bore so

as to ensure the orientation of the field substantially normal to the substrate plane. The magnetic field generated in such arrangements increases with the current applied to the coils(s) of the electromagnet, thereby providing for the application of magnetic fields which vary in time, for example in the form of a linear ramp (for current increasing linearly in time) or sinusoidally (for current varying sinusoidally as a function of time, i.e., an AC current).

Given the field-dependence of the number density within the planar assembly or array, an electromagnet configuration, via real-time control of the current, thus provides a means to reversibly adjust the number density of the assembly in real time. In a preferred embodiment, an AC current, with frequency in the range 10Hz to 10 MHz, and more typically in the range of 10Hz to 10kHz, is superimposed on a DC current, the amplitude of the AC current chosen to be small compared to the magnitude of the DC current: the former provides a temporal variation of interparticle distance within the assembly while the latter sets the average density. This induced density variation of the planar assembly will set up an electrokinetic response and local flow in the surrounding fluid medium. This magnetic field-induced flow has utility, for example by providing local mixing to enhance the kinetics of any reaction of interest occurring near the substrate and/or near the particle surface. In addition, spatially modulated magnetic fields can be produced by patterning the substrate with perm-alloy using methods known to the art.

The invention discloses a platform for the implementation of multistep bioanalytical assay procedures using the libraries of the composite particles of the invention to integrate multiplexed molecular interaction analysis with sample preparation and processing steps.

Sample preparation may include capture and separation of desired analytes from a given mixture, and sample processing may include any desired transformations carried out on captured analytes and/or binding agents. Analysis typically may include the detection and recording of indicators of the degree of molecular interaction between bead-displayed

5 binding agents and analyte molecules, an optical signature such as fluorescence or chemiluminescence representing a typical example of such an indicator; in addition, analysis may include the in-situ decoding the identities of binding agents associated with individual particles.

10 In each particular library of the present invention, a binding agent of interest in a desired assay is associated with a distinguishable tag in the form of a color-code. Of specific interest to the present invention are sub-libraries of encoded particles which enable real-time decoding, and hence the determination of the chemical identity of the binding agent. In the present invention, pre-selected binding agent are separately attached to the magnetic  
15 nanoparticles of the invention, to produce a sub-library of functionalized magnetic nanoparticles, and this sub-library is combined with a separately prepared sub-library of encoded particles serving as bead tags so as to uniquely associate to each functionalized magnetic nanoparticle contained in the first sub-library. This process provides unique flexibility in controlling the composition and possible permutations of functional groups and  
20 tags in the resulting library.

In one embodiment of the present invention, a library of the composite particles of the invention can be utilized in conjunction with standard microwell format and liquid handling robotics. The microwells contain a set of particles selected from the library in  
25 accordance with the requirements of the assay of interest. Multiplexed interaction analysis can be performed in one or more wells or other equivalent reaction vessels, preferably under conditions ensuring that the composite particles of the invention remain in suspension in order to achieve favorable reaction kinetics and maintain optimal mixing of the reaction. Each of the composite particles present in the reaction is able to interact with the same  
30 mixture of analyte molecules so as to facilitate the simultaneous formation of multiple

binding agent complexes in this multiplexed assay format in a manner described in the Examples set forth herein. Following completion of the molecular interaction and formation of binding agent complexes on the individual particles, within the set contained in each reaction vessel, the particles are analyzed and decoded, for example, by utilizing flow  
5 cytometry in a serial mode of analysis, to decode the particles and record assay signals one particle at a time, or by transferring one or sets of particles to substrates permitting the formation of planar assemblies for example by permitting the particles to settle under gravity.

10 In a preferred embodiment, assay integration is achieved in a microfluidic environment that eliminates the microwell format. As discussed below, sample capture, subsequent transformations of bead-displayed binding agents or analytes and real-time formation of random encoded assemblies of encoded particles subsequent to completion of the binding interaction, the latter permitting instant imaging detection in accordance with the  
15 READ process, are all integrated in a highly parallel assay format. A random encoded bead array, composed of the composite particles of the invention formed in real-time by application of a magnetic field in accordance with the present invention, provides a platform which can be used to perform multi-step assay sequences.

20 For purposes of illustration of the invention, four sets of steps (namely capture, transformation, post-transformation array assembly and random encoded array detection) can be carried as shown in Figure 22. These steps are further illustrated in the Examples that follow and provide the ability to perform an integrated bioanalytical assay. The assay protocol may be implemented in a microfluidic device of standard design, composed of one  
25 or more fluidic compartments connected by fluidic conduits and providing for the timed transport of aliquots of fluids using standard methods of pumping. In a preferred embodiment, sample capture and transformation are performed in a first compartment; following transformation, beads are transferred to a second compartment equipped with a magnetic field to complete post-transformation assembly and the READ process.

30

Sample of interest and a customized set of the composite particles are introduced into a first compartment and allowed to interact so as to permit capture of analytes from the sample to bead-displayed binding agents. Following application of a magnetic trap, one or more washing cycles are performed which involve the replacement of the content of the first  
5 compartment by aliquots of fresh buffer and finally, to initiate the transformation step, the introduction of an aliquot of reagents required for the transformation reaction, as described in the Examples provided herein.

The following describes in more detail the four steps of the process of the invention.

10

1. Capture

The analysis of biological samples typically begins with complex mixtures such a blood, serum or cell suspensions that contain not only the analytes of interest, be they  
15 antibodies, antigens, RNA, DNA or other biomolecules, but also a great variety of constituents which may interfere with the intended analysis. As with standard analytical chemical separation, it is generally desirable if not necessary to separate the analyte fraction of sample from the remainder. The suitability of certain magnetic particles for this purpose has been widely documented in the prior art, said particles, when used in the microwell  
20 format, generally requiring a high magnetic susceptibility to permit their collection and immobilization within reasonable time in magnetic field gradients which may be generated in a laboratory setting by use of permanent magnets. In this step, the capture of functional groups of interest, including, for example, genomic DNA fragments and the like to a first set of magnetic particles can be accomplished. The capture step is immediately followed by one  
25 or more washing cycles involving the introduction of fresh buffer to replace mobile (non-immobilized) constituents of the original mixture. Inductive elements also have been described to produce magnetic fields and field gradients within a miniaturized environment.

In certain instances, it may not be necessary to distinguish between different types of  
30 analytes of interest; examples include the extraction of total mRNA from cell lysate by

hybridization of the common poly-A tail sequence to oligo-dT displayed on standard magnetic particles or the affinity purification of a class of antibodies, say IgG, by binding of the Fc portion of the molecule to bead-displayed protein A, as in standard affinity purification.

5

However, in many instances, it is desirable or necessary to capture multiple analytes from a given sample in an analyte-specific manner. In the absence of a library of the composite particles of the invention, the sample require splitting into multiple aliquots, an additional step which not only has the potential to introduce inaccuracies such as pipetting error or contamination, but require the use of prohibitively small amounts of available sample. In contrast, libraries of the composite particles of the invention facilitate multiplexing by way of simultaneous capture in an analyte-specific manner. Examples include the sequence-specific capture of multiple mRNA targets of interest from cell lysate to matching bead-displayed capture sequences; the selection of multiple cDNA targets from a cDNA library; or the capture of multiple rare types of lymphocytes, identified by their respective repertoire of expressed cell surface antigens, to bead-displayed monoclonal antibodies. As described in Example 25, sample introduced in the specific capture step can be produced in a first, non-specific capture step involving, for example the magnetic nanoparticles of the invention.

20

In one embodiment of magnetic capture of the composite particles during wash cycles, permanent magnets known in the prior art may be employed to achieve temporary immobilization. Here, miniaturization of the assay environment ensures that particles always reside within a short distance of typically not more than 100  $\mu\text{m}$  from the nearest bounding surface of the reaction vessel, thereby reducing the time required to collect particles of given magnetic susceptibility from suspension into a magnetic gradient, or, conversely, to minimize the requisite magnetic susceptibility to ensure trapping within a given collection time, typically not more than 5 minutes and preferably not more than 0.5 minutes, by a magnetic field and field gradient of given strength. For the composite particles of the present invention, the magnetic susceptibility as determined by the constituents of the shell rather

30

than the core, and they are therefore particularly well suited to miniaturized assay environments.

One embodiment of the present invention may utilize designs of inductive elements known to the art for particle trapping at or near planar substrate surfaces. In a preferred embodiment, capture is performed within a first microfluidic compartment permitting application of magnetic fields. For example, a planar fluidic compartment may be sandwiched between two permanent magnets which are arranged to provide a sufficiently strong trap to hold particles against the two bounding surface of the compartment in the presence lateral flow up to a certain rate, but will release them in the presence of higher flow rates; equivalent magnetic field configurations also are readily produced by combination of solenoid magnets. A further alternative is provided by an axially symmetric configuration such as a capillary tube of 10-100  $\mu\text{m}$  diameter which may be inserted into the center of a quadrupolar field to provide trapping.

15

## 2. Transformation

The transformation of the captured analytes, or, in some cases relevant to DNA analysis, the analyte-mediated transformation of the bead-displayed probe itself represents an important processing step. Within the context of molecular biology, the most important processing steps involve several well studied enzyme-mediated modifications of RNA or DNA, notably including, in the former case, reverse transcription, and in the latter case, ligation, extension of bead-displayed probes (also known as primers) or amplification by cycling of extension reactions, most prevalently by application of polymerase chain reaction (PCR) protocols well known to the art. Specifically of interest to the present invention are implementations of on-bead RNA and DNA modification reactions. As with the capture step, multiplexing of reactions such as PCR using bead-tagged primers calls for the use of libraries of the composite particles of the present invention. An example is the solid-phase amplification of captured "particle-tagged" fragments. In the transformation step, "particle-tagged" DNA fragments are subjected to a set of "multiplex" transformation steps such as

30



for example PCR amplification. Another example is the polymerase-mediated extension of bead-displayed probe to produce a bead-displayed cDNA strand whose composition is complementary to the captured RNA target. In general, it also is possible to create solution-borne products as a result of the transformation which may then be captured to a second set  
5 of the composite particles of the invention as exemplified herein.

Captured protein analytes also may be transformed prior to further analysis. For example, proteins modified to contain a histidine derivative may be modified by chelation to nickel-functionalized reagents and tags using methods known to the art.

10

### 3. Post-Transformation Array Assembly

The present invention utilizes the magnetic field-induced formation of arrays comprising the composite particles of the invention as a means to produce random encoded  
15 assemblies and arrays of particles within the context of a highly bioanalytical parallel assay format in real time following completion of transformation. This process can produce planar assemblies of the composite particles of the invention in designated regions of the substrate surface to facilitate subsequent imaging of assemblies, and can produce such assemblies and ordered arrays under the widely varying conditions ensuring successful molecular interaction  
20 analysis and stability of the resulting binding agent complex, these conditions pertaining to buffer formulation (salt concentration, pH), presence of additional constituents such as surfactant or adjuvant, temperature and the like. Finite regions of the substrate can be delineated by interfacial patterning as described in the LEAPS process. In addition, spatially modulated magnetic fields can be produced by substrate patterning using, for example,  
25 permalloy in accordance with standard procedures.

The particles of the invention can be assembled into planar assemblies or ordered planar arrays in response to applied magnetic fields. For example, particles provided within suspension in a fluidic compartment bounded by two substantially planar surfaces that are  
30 arranged in a mutually parallel configuration in a sandwich geometry, when exposed to a

magnetic field oriented in a direction normal to the bounding surfaces, will form a planar assembly. For given particle magnetic susceptibility as controlled by the methods of the invention, ordered planar assemblies of characteristic number density will form in response to increasing magnetic field strength; in addition, linear strings of beads oriented normal to  
5 the substrate (and hence substantially parallel to the applied field) can be formed.

The application of suitably designed magnetic fields generated by a configuration of permanent magnets or electromagnets are well known in the art. Permanent magnets can be designed so as to produce the field strength required to realize the desired configuration of  
10 the assembly. Requisite magnetic field configurations can be produced by an electromagnet, for example in solenoid or Helmholtz configuration known to the art; the substrate can be introduced into the magnet bore or can be placed in immediate proximity to the coil(s) outside of the bore so as to ensure the orientation of the field substantially normal to the substrate plane.

15

In another embodiment, a magnetic trap of the type described in connection with the capture step can be employed to exchange the buffer employed for molecular interaction analysis for a second buffer that is optimized to realize conditions ensuring rapid array assembly by application of LEAPS.

20

#### 4. Random Encoded Array Detection

Once assembled, the random encoded array of the composite particles of the invention may be imaged to record assay signals and may be decoded to identify binding  
25 agents associated with individual beads within the array by the methods and procedures described herein.

Following formation of an array with the composite particles of the invention, the array provides a platform which can be used to read the results of the multi-sep assay  
30 sequences. The methods disclosed herein permit rapid customization of DNA or protein

arrays without the need for process redesign and avoid problems contributing to spot-to-spot as well as chip-to-chip variability. Furthermore, the particle array format permits chip-independent characterization of particles as well as optimization of assay conditions. In addition, multiple particles arrays can be formed simultaneously in discrete fluid  
5 compartments maintained on the same chip, permitting the concurrent processing of multiple samples.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for  
10 illustrative purposes and are not to be construed as limiting this invention in any manner.

## **EXAMPLES**

### **Example 1: Optically Programmable Array Formation**

15

As illustrated in Figure 9, LEAPS serves to simultaneously assemble multiple random encoded subarrays and to “drag-and-drop” these subarrays into separate, but proximate locations on the chip within a common, enclosed liquid environment. Two sets of beads (2.8  $\mu\text{m}$  Oligo-(dT)<sub>25</sub>, Dynal, Oslo, Norway), dispensed from separate reservoirs A  
20 and B, were simultaneously assembled into distinct subarrays within the same fluid; subarrays were then simultaneously placed into desired destinations as directed by spatially varying illumination profiles which were generated and projected onto the substrate by a PC-programmable illumination pattern generator (described in U.S. Serial No. 09/397,793, filed September 17, 1999, which is incorporated herein by reference in its entirety). This drag-  
25 and-drop operation reduced the separation between the two sub-arrays from approximately 250  $\mu\text{m}$  to 20  $\mu\text{m}$ . Beads were moved at 5 V<sub>pp</sub> at a frequency of 2 kHz; total power projected onto the substrate surface was  $\sim 5$  mW. The combination of chemical and spatial encoding permits a given set of chemical bead markers to be used multiple times and reduces the demands placed on either encoding dimension while facilitating the realization of large  
30 coding capacities.

### Example 2: Array formation on Patterned Surface

Illustrated in Figure 10 is an array of encoded beads assembled on a patterned silicon chip using an AC voltage of 1-2  $V_{pp}$  and a frequency of 100-150 Hz, applied across a 100  $\mu\text{m}$  electrode gap filled with an aqueous bead suspension; a thermal oxide ( $\sim 1000$  Å) on the substrate was patterned by etching the oxide to a thickness of 50-100 Å in a set of square features ( $\sim 30 \times 30 \mu\text{m}^2$ ) on 130  $\mu\text{m}$  centers; arrays of similar layout also can be produced in response to suitable illumination patterns. Each sub-array shown here contains approximately 80 beads coupled with anti-cytokine monoclonal antibodies. Carboxylate-modified polystyrene beads of 5.5  $\mu\text{m}$  diameter (Bangs Laboratory, Fishers, IN) were stained with a combination of two types of fluorescent dyes and were then functionalized with anti-cytokine-mAb. The assembly process ensures collection of all beads at the substrate surface. Bead encoding was as follows: IL-2 (Bright Red); IL-4 (Dim Red); IL-6 (Bright Green); M-CSF (Dim Green) and TNF- $\alpha$  (Yellow).

### Example 3: Formation of Arrays of Magnetic Nanoparticles

Colloidal particles exhibiting a finite diamagnetic susceptibility, when disposed on a planar substrate can be assembled into ordered arrays in response to increasing magnetic fields. Commercially available superparamagnetic particles (Dynal, Oslo, NO), dispersed from a fluid suspension onto the planar surface of the lower of two parallel bounding surfaces of a fluid cell ("sandwich" geometry), when exposed to a homogeneous axial magnetic field (oriented normal to the substrate plane), will form ordered assemblies. As a function of increasing magnetic field strength, and for given diamagnetic susceptibility of the particles as controlled by the manufacturing process known to the art, ordered planar assemblies and linear strings of beads oriented normal to the substrate can be formed. Permanent magnets can be designed so as to produce the field strength required to realize the desired configuration of the assembly. Requisite magnetic field configurations can be produced by an electromagnet in solenoid or Helmholtz configuration known to the art; the

substrate can be introduced into the magnet bore or can be placed in immediate proximity to the coil(s) outside of the bore so as to ensure the orientation of the field substantially normal to the substrate plane. Spatially modulated magnetic fields can be produced by patterning the substrate with permalloy using methods known to the art.

5

#### **Example 4: Formation of Random Bead Assemblies**

Aliquots of solution containing suspended beads were placed onto several distinct positions on a planar substrate of silicon capped with a thin silicon oxide layer (other  
 10 substrates may be used here). Beads were allowed to settle under gravity to form random assemblies. To delineate discrete positions on the substrate, one of the following two methods were used. According to the first method, a silicon gasket (of 250  $\mu\text{m}$  thickness), displaying a grid of multiple round holes of 1 mm or 2 mm diameter (Grace Bio-labs, Bend, Oregon) is placed on the hydrophilic surface to define microwells (of 0.25 to 0.5  $\mu\text{l}$  volume)  
 15 for multiple discrete samples of bead suspension. According to the second method, small aliquots of fluid containing beads (0.2  $\mu\text{l}$  to 0.5  $\mu\text{l}$  in volume) are directly placed onto a hydrophilic surface in one or more designated areas so as to ensure formation of discrete droplets; spacers are not needed in this case. As solvent evaporates (at room temperature or, for rapid drying, at elevated temperature (about 60  $^{\circ}\text{C}$ ), beads are left in random  
 20 positions on the substrate. DNA polymorphism reactions have been tested in assemblies formed in both manners. Optionally, beads settling under gravity may be immobilized by chemical capture layers provided on the substrate. An application of random bead assemblies to determine affinity constants in a multiplexed format is described in Example 6.

#### **25 Example 5: An Automated Chip-Scale array Manufacturing process**

As illustrated in Figure 11, the process involves liquid handling and pipetting of beads onto chips mounted in single-chip cartridges or multi-chip cartridges. Bead arrays are formed using methods such as those in Examples 1, 2 or 3., followed by array

immobilization and decoding. The resulting decoding images are stored for later use along with an optional chip ID (“bar code”).

### Example 6: Determination of Affinity Constants by Post-Assay Analysis of Bead Assemblies.

Quantitative binding curves for the cytokines TNF- $\alpha$  and IL-6. Binding curves were generated by performing sandwich immunoassays using chemically encoded beads in suspension, said suspensions being confined to one or more reaction compartments delineated on-chip, or in one or more reaction compartments off chip. By completing the reaction with beads maintained in suspension, assay kinetics similar to homogeneous assays can be attained. Following completion of the binding reaction, beads were assembled on chip to permit multiplexed quantitative image analysis. Random assemblies prepared according to Example 4 or ordered bead arrays prepared according to Example 1 or 2 may be used. An advantage of ordered, dense assemblies produced by the methods of Examples 1 or 2 is the higher spatial density and higher assay throughput attained by processing a greater number of beads.

As an illustration, Figure 12 displays quantitative binding curves for TNF- $\alpha$  and IL-6, obtained from randomly dispersed beads. A commercial-grade 8-bit video-CCD camera (Cohu, San Diego, CA) was used in a mode permitting multi-frame integration. The range of concentrations of antigen used in the two assays was 700 fM to 50 pM for TNF- $\alpha$  and 2 pM to 50 pM for IL-6. At each concentration, the number of molecules bound per bead was estimated by comparison with calibration beads coated with known quantities of Cy5.5-labeled BSA per bead; requisite adjustments were made to account for differences in fluorescence quantum efficiency between labeled secondary antibodies and BSA.

This format of analysis permits the determination of the affinity constant,  $K_A = [LR]/([R_0-LR][L])$ , where, in accordance with the law of mass action,  $[LR]$  denotes the number of receptor-ligand pairs per bead and  $[L]$  denotes the solution concentration of ligand. By specifying the number of beads per ml,  $n_B$  and specifying a value for  $[R_0]$  in terms

of the number of receptors per bead, theoretical binding curves, computed for given  $K_A$ , are compared to a plot of the number of bound molecules per bead as a function of bulk ligand concentration. The absolute number of ligands bound per bead may be determined for given bulk concentration by measuring the mean fluorescence intensity per bead and referencing  
 5 this to the fluorescence intensity recorded from calibration beads included in the array.

The estimated number of molecules bound per bead is compared to theoretical binding curves derived from the law of mass action. The three curves shown correspond to values of the affinity constant,  $K_A$ , of  $10^{11}$ /molar,  $10^{10}$ /molar and  $10^9$ /molar, respectively. The initial number of antibodies per bead,  $R_0$ , equals  $2 \times 10^5$ /bead and  $n_B =$   
 10  $10^5$ /ml. Each data point represents the average of three replicates, with an assay-to-assay variation of  $< 45\%$ . Setting the assay sensitivity to correspond to that level of fluorescence which yields a signal-to-noise level of unity in the assay images, the sensitivity of the cytokine assays characterized in Figure 12 is set at  $\sim 2,000$  bound ligands/bead, corresponding to respective detected concentrations of 700 fM for TNF- $\alpha$  and 2 pM for IL-  
 15 6.

While commercial ELISA kits use enzymatic amplification to enhance sensitivity, at the expense of additional complexity relating to assay conditions and controls, our bead array assay format, even without enzymatic amplification, our on-chip assay format permits monitoring of cytokines at circulating levels (Normal TNF- $\alpha$  level in serum is 50-  
 20 280 fM and normal IL-6 level in serum is 0-750 fM. [www.apbiotech.com/technical/technical\\_index.html](http://www.apbiotech.com/technical/technical_index.html)), providing a dynamic range which approaches that of standard, i.e. amplified single-analyte ELISA assays (Assay kits of R & D Systems and Amersham (not the recent High-Sensitivity assays). Further improvements at hardware and software levels are possible.

25

### **Example 7: Genotyping by Polymorphism Analysis**

To illustrate the application of the present invention to the implementation of genotyping, Figure 13 shows the design of the assay in which five pairs of 20-mer binding  
 30 agents corresponding to four polymorphic regions of a gene were coupled to color-encoded

beads. The pairs of binding agents  $\alpha 1$ ,  $\alpha 2$  and  $\beta 1$ ,  $\beta 2$  each display a single nucleotide difference in their respective sequences; the pair  $\delta 3$ ,  $\delta 4$  displays a difference of three nucleotides, the binding agents in the set  $\gamma 1$ ,  $\gamma 3$ ,  $\gamma 3$ ,  $\gamma 4$  display small insertions and deletions. The ten binding agents were are divided into two subgroups of five which were

5 incorporated into two subarrays. In this example, there are several hundred beads for each type. Following bead immobilization, an on-chip hybridization reaction was performed in TMAC buffer (2.25 M tetramethylammonium chloride, 37 mM Tris pH 8.0, 3 mM EDTA pH 8.0, and 0.15% SDS) at 55°C for 30 min. The analyte is a 254-base PCR fragment produced from a patient sample and fluorescently labeled at the 5'-prime end with BODIPY

10 630/650 (Molecular Probes, Eugene, OR). Image acquisition was performed after replacing the assay buffer with fresh TMAC buffer.

Figure 14 shows decoding and assay images for one subarray. Each bead shown in the assay image obtained after hybridization is analyzed to determine fluorescence intensity

15 and bead type; as with the cytokine assay, the latter operation compares assay and decoding images using a template matching algorithm. Figure 15 displays the resulting intensity histograms for each bead type: in these histogram plots, the horizontal axis refers to relative signal intensity from 0 to 1 and the vertical axes refer to bead numbers. The histograms show that most of the beads displaying probe  $\alpha 1$  bind no analyte while most of the beads

20 displaying probe  $\alpha 2$  exhibit significant binding; the mean signal level of  $\alpha 2$ -beads exceeds that of  $\alpha 1$ -beads by a factor of  $\sim 3.2$ , indicating that analyte contains DNA sequences complementary to  $\alpha 2$  but not  $\alpha 1$ . For the patient sample presented here, the histogram indicates a genotype of the analyte DNA characterized by complementarity to binding agents  $\alpha 2$ ,  $\beta 2$ ,  $\gamma 3$ ,  $\gamma 4$  and  $\delta 4$  in the polymorphic region of the gene.

25

#### **Example 8: Gene Expression Analysis: cDNA Fragments**

The method of the present invention has been used to fabricate arrays composed of beads displaying oligonucleotides as well as DNA fragments (e.g., up to  $\sim 1,000$  bases in

30 length). Strands were biotinylated at multiple positions by nick-translation and were attached



to streptavidin-functionalized beads (M-280, Dynal, Oslo, NO). Arrays were formed using an AC voltage of 800 Hz at  $10V_{pp}$ .

### Example 9: Looped Probe Design for Universal Labeling

5

A looped probe design in Figure 16 takes advantage of fluorescence energy transfer to obviate the need for labeled target. As with the molecular beacon design (S. Tyagi, D. P. Bratu, F. R. Kramer, *Nature Biotech.* 16, 49-53 (1998)), the probe in Figure 16 assumes two different states of fluorescence in the closed loop and open loop configurations, but in contrast to the molecular beacon contains a portion of its binding motif within the stem structure to permit molecular control of stringency in competitive hybridization assays.

### Example 10: Quantitative Multiplexed Profiling of Cytokine Expression

Figure 17 displays a pair of assay and decoding images recorded from a single random array in a multiplexed sandwich immunoassay. An array containing five distinct types of beads, each displaying a monoclonal anti-cytokine antibody (mAb), was exposed to a sample solution (such as serum) containing two cytokine antigens (Ag). Subsequent addition of Cy5.5-labeled secondary antibodies (pAb\*) results in the formation of ternary complexes, mAb-Ag-pAb\*. The on-chip immunoassay was performed by adding 300  $\mu$ l of sample with 7 nM cytokines in assay buffer (20 mM NaPi pH 7.4, 150 mM NaCl, 10 mg/ml BSA) to the bead array immobilized on the chip, and allowing the reaction to proceed at 37°C for one hour. The buffer was replaced by adding 12 nM solution of labeled secondary antibodies in assay buffer. After one hour of incubation at 37°C, fresh buffer was added on top of the chip and image acquisition was performed. Antibodies and antigens used in the assays were obtained from R&D Systems (Minneapolis, MN); the secondary antibody was labeled with Cy5.5 using a standard kit (Amersham Pharmacia Biotech, Piscataway, NJ).

The decoding image Figure 17B shows five types of beads in a false-color display with the same encoding pattern as that of Figure 10. All beads are of the same size (5.5  $\mu$ m

diameter); the apparent difference in the size of beads of different types in the decoding image is an artifact reflecting different internal bead staining levels and "blooming" during CCD recording of the decoding image. Comparison (using the image analysis methods disclosed herein) of the decoding image with the assay image in Figure 14A reveals that

5 active beads, of yellow and bright green types, captured TNF- $\alpha$  and IL-6, respectively. This assay protocol has been extended to the following set of twelve cytokines: IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, TGF- $\beta$ 1, IL-12, EGF, GM-CSF, M-CSF, MCP-1 and TNF- $\alpha$ . The on-chip immunoassay requires no additional washing other than changing reagent solutions between assay steps. Comparison between assay and decoding images shows that two different

10 cytokines were present in the sample, namely IL-6 and TNF- $\alpha$ . The pre-formed arrays described in this example also permit the determination of affinity constants in a manner analogous to the analysis described in Example 6.

#### **Example 11: Aptamers for Protein Profiling**

15

Aptamers may be selected from large combinatorial libraries for their high binding affinities to serum proteins (L. Gold, B. Polisky, O. Uhlenbeck, M. Yarus, *Annu. Rev. Biochem.* 64: 763-797. (1995)). Random encoded arrays of aptamer-functionalized beads would serve to monitor levels of serum proteins; correlations in binding patterns on the array

20 (see also Example 10) may serve as a phenotype of disease states.

#### **Example 12: Mixed DNA -Protein Arrays**

Of significant interest to genomic functional analysis is the fact that the method of

25 the present invention accommodates protein and DNA arrays without change in array manufacturing methodology. Specifically, mixed arrays composed of beads displaying DNA and corresponding proteins can be used to analyze the gene and gene product within the same fluid sample.

This has been demonstrated for a combination of immunoassay and DNA

30 hybridization. For example, a mixed array composed of beads functionalized with anti-

cytokine monoclonal antibodies (mAb) and with oligonucleotides was produced. Two sequential assays were performed on this single chip. First, an immunoassay was performed in accordance with the protocol described in Example 10 . Following completion of the on-chip immunoassay, image is acquired and the DNA analyte was added to the hybridization buffer (2x SSC, 1x Denhardt's) at a final concentration of 20 nM and allowed to react at 37°C for 1 hr. Fresh hybridization buffer was added to the chip and image acquisition was performed to record of the additional hybridization assay.

### Example 13. Affinity Fingerprinting

10

The analysis of receptor-ligand interactions relevant to prior art methods assumes ideal specificity. That is, only the ideal situation is considered of a single ligand present in solution reacting with its matching receptor and vice versa. However, in most multiple assay systems, a considerable level of cross-reactivity may exist. That is, any single ligand may associate with several receptors, while any single type of receptor may have a finite affinity towards more than one ligand.

The present invention includes a model that is developed to analyze multiplexed READ assays for such a system under the following assumptions: each of these reversible reactions is characterized by its own affinity constant; no reaction occurs between the bulk species; there is no interaction between the complexes formed on the surface. These assumptions can be relaxed, at the expense of increasing the complexity of modeling, by accounting for reactions in the bulk and between the surface species. The standard reaction-diffusion equation for single receptor-ligand pair formation [R. W. Glaser, Anal. Biochem. 213, 152-161 (1993)], is generalized to allow for multiple reactions at each bead surface:

$$\frac{\partial [L_i \cdot R_j]}{\partial t} = k_{on,ij} [L_i] \left( [R_{j,0}] - \sum_{n,m} [L_m \cdot R_n] \right) - k_{off,ij} [L_i \cdot R_j] \quad \forall i, j, L_i \equiv L_i(t, x, 0) \quad (2)$$

The first term on the right of Eq. (1) describes the association of ligands and receptors into complexes and involves of concentration of free sites on the surface. The second term describes the disassociation of complexes by way of release of ligands, thereby freeing up receptor sites for further reaction. Since a maximum of  $(i \times j)$  bimolecular complexes can form, there could be as many boundary conditions generated from the above equation. For the equilibrium case, the left hand-side of Eq. (1) is set to zero, and the matrix of coaffinities,  $[K_{ij}] = k_{on,ij}/k_{off,ij}$ , can then be defined to accommodate cross-reactivities between multiple species in the bulk and on the surface. In a batch reactor under equilibrium conditions, we may solve the system of differential equations to obtain the number of molecules of each ligand bound on beads of each type.

$L_1$	Ligand concentration	10 pM
$L_2$	Ligand concentration	100 pM
$R_{01}$	Initial receptor concentration	$1 \times 10^4$ /bead
$R_{02}$	Initial receptor concentration	$1 \times 10^4$ /bead
$n_{B1}$	Bead number density	$1 \times 10^4$ /ml
$n_{B2}$	Bead number density	$1 \times 10^4$ /ml
$[K]$	Coaffinity matrix	$[1 \times 10^{11} \ 1 \times 10^9$ $1 \times 10^8 \ 1 \times 10^{11}]$ l/mole

As an illustrative example, the ligand distribution has been calculated (from the model in Eq (1)) for a reference set of two ligands and two types of receptors immobilized on two different sets of beads. The coaffinity matrix is assumed known for each ligand-receptor combination in the reference set; to investigate the detection of a third ligand, it is assumed here that diagonal elements of the  $2 \times 2$  matrix,  $[K_{ij}]$ , are large compared to off-diagonal elements. The presence of a third ligand in the reactor alongside the two original ligands perturbs the equilibria between the various complexes and the reactants in the reference system, and for ligand molecules tagged with fluorescent labels, the intensity observed from the perturbed system differs from that observed in the reference case.

Figure 18A shows the reference case in which the concentrations and coaffinity matrix were set to the values shown in the accompanying table; the bead intensity was defined on a linear scale of 0 - 255, the latter representing the intensity of the brightest beads. Figure 18A shows the contribution of each ligand to the bead intensity. Due to the  
 5 lower concentration of  $L_1$ , the intensity of Bead 1 is less compared to Bead 2, cross-reactivities are essentially undetectable.

Next, the system was perturbed with a third ligand, taking the concentration  $L_3$  to be 1 pM and assuming that the new ligand has considerable amount of cross-reactivity with  
 10 each of the receptors;  $K_{3,1} = 1 \times 10^{11} / M$ ,  $K_{3,2} = 1 \times 10^{10} / M$ . Calculation of the fluorescent intensity of each bead in the presence of the third ligand yields the pattern in Figure 18A which reveals an increase in the intensity of Bead 1 due to the third ligand, while leaving the intensity of Bead 2 unaffected due to the higher concentration of  $L_2$  in the system and the lower affinity of  $L_3$  to  $R_2$ . Thus,  $L_3$  may be detected under the condition that it has a  
 15 relatively high affinity to one of the receptors and is in significant amount compared to the competing ligand.

The evaluation of the coaffinity matrix (and comparison with theoretical modeling as disclosed herein) under conditions in which a mixture of ligands is permitted to interact with  
 20 a multiplicity of receptors arranged in a random encoded bead array format provides a methodology to establish a characteristic feature set of cross-correlations in the mutual competitive binding affinities of multiple ligands and receptors. These co-affinities provide a robust means to characterize receptor-ligand binding equilibria by their affinity fingerprinting patterns. Deviations from well-defined reference cases also permit detection of “perturbing”  
 25 ligands in solutions.

#### **Example 14. Multiplexed Analysis of Reaction Kinetics**

As illustrated in the foregoing examples, extensive washing generally is not required  
 30 to discriminate beads from a background of solution fluorescence. Consequently, assay

image sequences may be recorded in a homogeneous assay format to document the evolution of a binding reaction and to determine kinetic data for each of the binding reactions occurring.

- 5 Homogeneous binding assays may be performed in simple “sandwich” fluidic cartridges permitting optical microscopic imaging of the bead array and permitting the introduction of an analyte solution into a chamber containing a random encoded array of beads. More generally, the array also may be exposed to an analyte or other reaction mixtures under conditions of controlled injection of fluid aliquots or continuous flow of
- 10 reactants or buffer. Using theoretical modeling, optimal combinations of relevant performance control parameters of this bead array reactor may be identified to minimize the time to equilibration or to maximize the portion of analyte captured by the array [K. Podual and M. Seul, TM KP-99/02]. Flow rate can be controlled by any of a number of available pumping mechanisms [M. Freemantle, C&EN, 77: 27-36].

15

Table - List of parameters used in simulations (Figure 18)

	Parameter, units	Value
	Initial Receptor Coverage $c_{R,0}$ , moles/m <sup>2</sup>	$8 \times 10^{-9}$
20	Vol Flow Rate, $Q$ , $\mu\text{l/s}$	1.0
	Diffusivity, $D$ , cm <sup>2</sup> /s	$1 \times 10^{-7}$
	ON-Rate, $k_{on}$ , /(M s)	$1 \times 10^5$
	Affinity Constant, $K_A$ , /M	$1 \times 10^{11}$
	“Sandwich” Reactor Gap Size $H$ , mm	0.1
25	Reactor Length, $L$ , mm	10
	Reactor Width, $W$ , mm	10

The analysis of image sequences permits kinetic data to be generated from which ON-rates and OFF-rates are determined with the aid of a theoretical model of the reaction-

diffusion kinetics of the type illustrated in the foregoing example in Figure 19. Figure 19A displays stages in an adsorption-desorption cycle involving solution-borne analytes and a bead array immobilized at the bottom of a “sandwich” reaction chamber. The first panel depicts the initiation of the adsorption process; the second panel depicts the state of the reactor close to equilibrium when most of the beads have reached equilibrium; the last panel depicts the state of the reactor under the desorption cycle in which ligand-free fluid is injected and adsorbed molecules desorb from the bead surface. Figure 19B displays the adsorption-desorption kinetics of a single receptor-single ligand system obtained by numerical solution of a reaction-diffusion system for a single type of receptor-ligand reaction; two cases of different concentrations of ligand are shown. Parameters used in the simulation are listed in the accompanying Table.

In contrast to prior art methods [D. G. Myszka, *Curr. Opin. Biotechnol.* 8: 50-57.], the present method relies on imaging and permits multiplexing. In addition, generalized models of the type introduced in Example 6 permit the analysis of complex binding kinetics for multiple simultaneous receptor-ligand interactions even in the presence of cross-reactions between multiple ligands and receptors.

The ability to monitor reaction kinetics in an array format will enable several approaches to enhancing the specificity of receptor-ligand or binding agent-analyte interactions in complex mixtures. For example, temperature programming may be invoked to enhance the specificity of DNA hybridization reactions. Similarly, the stringency of conditions applied to a hybridization reaction may be varied while the array response is being monitored; for example, hybridization may be conducted in a hybridization buffer under conditions leading to excess “non-specific” binding; specificity is enhanced by switching to a wash buffer of increasing stringency while monitoring the array response.

#### **Example 15. Multi-Step Assay Sequences Using Encoded Arrays of Magnetic Particles**

30

Methods and apparatus using biochemically functionalized super-paramagnetic particles for sample preparation in molecular and cellular biology and for a variety of enzyme-catalyzed on-bead reactions have been described [“Biomagnetic Techniques in Molecular Biology”, Technical Handbook, 3<sup>rd</sup> Edition, 1998, Dynal, Oslo, NO]. These bead-  
5 based methods can be combined with the Random Encoded Array Detection format of the present invention to implement multi-step on-chip assay manipulations.

For example, Figure 20 illustrates the integration of a sequence of steps in a miniaturized format for multiplexed genotyping using a single chip with multiple  
10 compartments. First, cells are captured from a patient sample by affinity selection using functionalized magnetic beads, cells are lysed electrically or chemically in a first compartment, and genomic DNA is captured to the surface of a multiplicity of magnetic beads by non-specific binding; next, beads are collected by magnetic force into a second compartment which is in fluidic contact with the first compartment, within which the beads  
15 and DNA are washed with desired buffers; next, beads are further transferred to a location where PCR is performed using bead-coupled DNA as a template; multiple PCR strategies known in the art are available for this step (F. Fellmann, et.al., Biotechniques, 21:766-770); next, PCR products released into are captured by hybridization to a pre-assembled random encoded array displaying binding agents that are specific to different polymorphism targeted  
20 by the PCR amplification.

The use of encoded magnetic particles in conjunction with the optical programmability of LEAPS confers the ability to form reversibly immobilized arrays and to conduct programmable multi-step assay sequences under conditions in which beads are used  
25 in suspension when this is most favorable, for example to enhance reaction kinetics, and arrays are formed in real-time when this is most favorable, for example to provide a highly parallel format for imaging detection and assay read-out.

For example, as illustrated in Figure 21, the following sequence of steps could be  
30 integrated in a miniaturized format for the formation of a cDNA bead array. First, a pool of



encoded magnetic beads, each bead type displaying a gene-specific probe, is introduced to an mRNA pool, and mRNA molecules are hybridized to their corresponding beads; next, on-bead reverse transcription (RT) is performed using bead-attached mRNA as template [E. Horenes, L. Korsnes, US 005759820]; next mRNA is released from the beads; next beads  
 5 are directed to the surface of a custom-designed chip and a cDNA bead array is formed using LEAPS. Such an array could serve to display binding agents in a gene profiling experiment using another set of mRNA as the target. Alternatively, the cDNA array could be analyzed for its own expression by applying a pool of labeled DNA binding agents to profile the genes of interest within the array.

10

**Example 16. Synthesis of Super-Paramagnetic Iron Oxide  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> (maghemite) particles**

The synthesis was carried out in reversed micellar solutions composed of the anionic  
 15 surfactant, bis(2-ethylhexyl)sodium sulfosuccinate (AOT) and isooctane (Kommareddi et al., Chem. Mater. 1996, 8, 801-809) obtained from Aldrich Chemical Co., Milwaukee, WI. Stock solutions of 0.5M AOT were used in preparing the reversed micellar solutions containing the reactants FeSO<sub>4</sub> (Sigma Chemical Co., St. Louis, MO) and NH<sub>4</sub>OH (Sigma Chemical Co., St. Louis, MO). Specifically, 0.45ml of 0.9M FeSO<sub>4</sub> was added to 5 ml of  
 20 0.5M AOT in isooctane, separately 0.45ml of NH<sub>4</sub>OH was added to 5 ml of 0.5M AOT in isooctane. The reaction was initiated by adding the NH<sub>4</sub>OH reversed micellar solution to the FeSO<sub>4</sub> reversed micellar solution under vigorous stirring. The reaction was allowed to proceed for ~2-3hrs and then the solvent was evaporated at ~ 40°C to obtain a dry surfactant iron oxide composite. This composite was re-dispersed in the organic solvent of  
 25 choice to give a deep red colored transparent solution.

**Example 16a. Synthesis of Magnetic Nanoparticles with a Functional Site.**

This Example illustrates the creation of a functional site on a magnetic nanoparticle.  
 30 A surfactant was dissolved in oil to obtain a reverse micellar solution which was used to

synthesize the magnetic nanoparticles of Example 16. The resulting reaction mixture was dried to obtain a dry surfactant past which was re-dispersed in an oil of choice. An aqueous solution of monomer(s) and or preformed polymer(s), a cross-linking agent and initiator were added. The mixture was subjected to a polymerization step, and the polymerized reaction product was dried. The dried mass was dispersed in aqueous buffer; and the methods can be combined with READ to implement multi-step on-chip assay manipulations.

### Example 16b. Coupling of a Biomolecules

10

This Example illustrates the coupling of a biomolecule to the magnetic nanoparticles of the invention. The magnetic nanoparticle of Example 16a can be further functionalized to perform an assay of interest by binding to the functional site on the magnetic nanoparticle certain molecules such as for example DNA (oligonucleotides) or RNA fragments, peptides or proteins, aptamers and small organic molecules. The binding of such molecules can be performed using processes known in the art, for example, using one or several coupling reactions (See, e.g, G. T. Hermanson, Bioconjugate Techniques (Academic Press, 1996); L. Illum, P. D. E. Jones, Methods in Enzymology 112, 67-84 (1985)). Attachment of the molecule of interest to the functional site generally requires a one-step or two-step reaction which can be performed in parallel using standard liquid handling robotics and a 96-well format to covalently attach any of molecules to the functional site on the magnetic nanoparticle.

Specifically, this Example illustrates a method of attaching a probe (for example a protein), to the functional site of a magnetic nanoparticle of the invention, using well-established carbodiimide chemistry. In a 2 ml vial an aliquot containing 10 mg of carboxylate functionalized magnetic nanoparticle was mixed with 1ml 10mM borate buffer (pH =8.5). The resulting particles were then separated magnetically using a permanent magnet separator and the supernatant was siphoned off. The separated pellet was washed two times (using the same protocol as above) in 0.1M MES buffer (pH = 4.5) and finally re-suspended in

600µl of the same . In a separate vial 3 mg of Neutravidin (a biotin binding protein, Pierce Chemicals, Rockford, IL) was dissolved in 300µl of the MES buffer and the solution was slowly added to the suspension of the magnetic charged particle. The suspension is sonicated using a probe sonicator. Following sonication, EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) (Aldrich-Sigma, Milwaukee, WI) solution (200mg/ml) was added . The mixture was allowed to react for 2 hours at room temperature, following which the resulting magnetic nanoparticles were magnetically separated, washed one time with a coupling buffer, two times in a borate buffer and resuspended and stored in a storage buffer (PBS pH = 7.4, 0.1%(w/v) BSA, 0.5%(w/v) Tween 20, 10mM EDTA and 0.02% (w/v) NaN<sub>3</sub>) at 2-8°C.

#### **Example 16c. Preparation of Composite Particles.**

This Example illustrates the production of encoded magnetic articles. Encoded particles were reacted with the magnetic nanoparticles of Example 16b. In a more general embodiment, super-paramagnetic polymer nanoparticles may also be used (such as those obtained from MACS microbeads, Miletnyi Biotech Inc, Auburn, CA; Captivate ferrofluid particles, Molecular Probes, Eugene, OR; Nanomag particles, Micromod, Rostock, Germany).

20

Figure 23 illustrates a sub- library of 12 types of encoded magnetic particles. The encoded particles were synthesized using 3.2 µm diameter cross-linked polystyrene particles with tosyl surface groups (Bangs Labs, Fishers, IN) and two hydrophobic pyrromethane dyes which were each introduced by swelling and bulk staining. The dyes were individually introduced to obtain four intensity levels, and were mixed in four nominal molar ratios. Following, 100µl of a 1% solution of the colored Latex ( in PBS) was mixed with 100µl or 200µl of Captivate ferrofluid (streptavidin conjugate, 200nm diameter, used as supplied) (Molecular Probes, Eugene, OR) or 100µl of Nanomag dextran coated. Streptavidin functionalized magnetite particles 130nm diameter were used as supplied) (Nanomag particles, Micromod, Rostock, Germany). The whole reaction volume is made up to 500µl

using phosphate buffer pH 7.8 and the coupling reaction is allowed to proceed overnight. The resulting particles are separated magnetically washed 1x, resuspended and stored in storage buffer (PBS pH = 7.4, 0.1%(w/v) BSA, 0.5%(w/v) Tween 20, 10mM EDTA and 0.02% (w/v)  $\text{NaN}_3$ ) at 2-8°C.

5

#### **Example 16d. Controlling the Content of Metal Oxide**

This Example illustrates the method by which the content of the metal oxide can be controlled to impart on the magnetic moment of the encoded magnetic particle of the invention. This can be accomplished by varying the number of magnetic nanoparticles which can bind to the surface of the encoded particle. For ease of monitoring, and by way of illustration, instead of magnetic nanoparticles, Fluoronanogold, a fluorescein-gold nanoparticle - streptavidin conjugate (supplied from Nanoprobes, Yaphank, NY) was used to monitor the number of particles. The reagent contained ~ 0.08mg/ml of streptavidin and was used as received.

In this Example, 0.5mg of biotin functionalized 3.36  $\mu\text{m}$  diameter polystyrene beads (Spherotech, Libertyville, IL) was suspended in 500 $\mu\text{l}$  of 20mM phosphate buffered saline (150mM) at pH 7.4 with 0.1% BSA. Varying amounts of Fluoronanogold reagent were added to the bead solution and the streptavidin-biotin binding reaction allowed to proceed for 1 hour at room temperature, following which the microparticles were separated by centrifugation and supernatant removed. Figure 24 shows the fluorescence intensity from the particles bound on the surface as a function of the amount of Fluoronanogold reagent added.

#### **Example 17. Synthesis of Fluorescently Colored and Magnetic Polymer Bead Composites**

A stock solution of hydrophobic fluorescent dye and the iron oxide particles was made by re-dispersing the dried magnetic composite and the dye in the solvent of choice, for example a  $\text{CHCl}_3$  (Aldrich Chemical Co., Milwaukee, WI) or  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  mixture

(70/30 (v/v)) (Aldrich Chemical Co., Milwaukee, WI). A predetermined amount of polymer beads was washed thoroughly in methanol (3x) and then evaporated dry. Simultaneous incorporation of the fluorescent dye and the iron oxide nanoparticle was achieved by swelling the beads in organic solvent/nanoparticle/dye mixture. The swelling process was  
5 completed within ~ 1hr. Following this the polymer beads were separated by centrifugation and washed with methanol(3x) followed by isooctane(2x) and then methanol(2x) and finally redispersed in 0.2% SDS –DI water solution.

#### **Example 18: Formation of Arrays by Application of a Magnetic Field**

10

The composite particles of the invention when exposed to a homogeneous axial magnetic field (oriented normal to the substrate plane), can be arranged in 2D array format. As a function of increasing magnetic field strength which is dependent on the paramagnetic susceptibility of the composite particle, planar assemblies can be formed. Permanent  
15 magnets can be designed so as to produce the field strength required to realize the desired configuration of the assembly. Requisite magnetic field configurations can also be produced by an electromagnet in solenoid or Helmholtz configuration known to the art; the substrate can be introduced into the magnet bore or can be placed in immediate proximity to the coil(s) outside of the bore so as to ensure the orientation of the field substantially normal to  
20 the substrate plane. Spatially modulated magnetic fields can be produced by patterning the substrate with perm-alloy using methods known to the art.

In this Example, three differently sized composite particles with wherein the encoded particles are 3.2 microns, 6.9 microns and 10.9 microns were synthesized as described  
25 herein, as expected, the magnetization of the composite particles increased with increasing particle size.

Figure 25 shows a 2D geometry of 3.2 microns composite particle exposed to a magnetic field of approximately 1000 Gauss produced using a permanent magnet.

30

### Example 19: Two-Dimensional Colloidal Arrays: Formation of Magnetic “Wigner” Crystals

This Example illustrates the ability to manipulate the composite particles of the invention. Exemplification is provided by the manipulation of superparamagnetic particles suspension such as superparamagnetic Oligo(dT)<sub>25</sub> microparticle (DynaL, Lake Success, NY). Suspension of these particles are formed by mixing the particle at a concentration of  $\sim 5 \times 10^7$  beads /ml) in 0.01mM saline, containing 0.05% triton-X100 (Sigma-Aldrich, Milwaukee, WI) as a stabilizer. The magnetization of the particles is completely reversible and, at low field strengths, is proportional to the external field through the effective volumetric magnetic susceptibility ( $= 0.192$ ). The suspensions are held on a microscope stage in a sandwich cell formed by a silicon electrode and an ITO coated glass electrode separated by an adhesive spacer  $\sim 100\mu\text{m}$  thick. A nominally uniform magnetic field is generated in the sample by a coil of copper-wire placed underneath the sandwich cell. After application of the magnetic field the evolution of the suspension structure is recorded with a CCD camera attached to a VCR and the images digitized for further analysis. Figure 26 shows the experimental setup and Figure 27 shows a series of snapshots illustrating the formation of 2-dimensional magnetic arrays.

When a magnetic field is applied, the particles acquire magnetic moments  $m = \frac{4}{3}\pi a^3 \mu_0 \chi H$ , where  $a$  is the particle radius,  $\mu_0$  the magnetic permeability of vacuum, and  $H$  is the external field.

### Example 20: Streptavidin-biotin Binding Assay Using Fluorescent Magnetic Microparticles

In this Example, to 100  $\mu\text{l}$  of a suspension of the composite particles of the invention, which have been functionalized with neutravidin as described in Example 16c (1%) solids is added to a 1.5ml vial and the suspension diluted with 900 $\mu\text{l}$  of PBS with 0.01% (w/v) of Tween-20 (PBST). The composite particles of the invention are mixed by

vortexing and then separated magnetically and the supernatant aspirated off. The pellet is resuspended in 980  $\mu$ l of PBS. 20 $\mu$ l of a biotin-Oligo(dT)<sub>5</sub>-CY5.5 (IDT, Coralville, IA) at a concentration (26.7ng/ml). The mixture is incubated for 30 minutes at room temperature. Following this the particles of the invention are separated magnetically and washed 2X in  
 5 PBST and resuspended in 1ml of PBST. The particles of the invention are then assembled on a chip and their surface fluorescence estimated using the method previously described. The results are shown in Figure 28. For purposes of comparison, the encoded particles of the invention in the absence of the magnetic nanoparticles of the invention, but having covalently attached thereon neutravidin were also reacted to the biotinylated probe using the procedure  
 10 described in this Example.

#### **Example 21. On-chip Hybridization of Target Molecules**

This Example relates to on-chip hybridization of target molecules to oligonucleotides  
 15 probes immobilized to the composite particles of the invention. Biotinylated oligonucleotides with known base sequence were first coupled to a given type of color-coded composite particles that have been previously coated with Neutravidin on the surface. The coupling reaction was carried out in 0.1 ml coupling buffer (150 mM NaCl, 0.05 mM ethylenediamine tetra-acetic acid (EDTA), 0.5% bovine serum albumin, 0.5 mM Tris-HCl, and 100 mM  
 20 sodium phosphate, pH 7.2) with 0.4  $\mu$ M biotinylated oligonucleotides, and approximately  $6.7 \times 10^5$  particles. The coupling reaction mix was incubated at room temperature for 30 min with vortexing. Upon completion of the coupling reaction, the particles were collected by centrifugation. The unreacted sites of the NeutriAvidin on the surface were blocked by using 0.1% biotin in 150 mM NaCl and 100 mM sodium phosphate, pH 7.2 with 0.05%  
 25 Tween-20. The blocking was carried out at room temperature for 20 min with vortexing. After blocking, the particles were washed with 0.2 ml of 150 mM NaCl and 100 mM sodium phosphate, pH 7.2 with 0.05% Tween-20. The foregoing procedure can be utilized to couple any biotinylated oligonucleotides of interest to different types of Neutravidin-coated particles of the invention.

Several types of color-encoded particles of the invention were coupled with biotinylated oligonucleotides with known sequences and were combined into one tube for assembly of arrays on silicon chips. The arrays thus formed were then used for on-chip hybridization of peptide nucleic acid (PNA) oligomers to specific complimentary oligonucleotides previously coupled to the micro particles of the invention. Specifically, hybridization was carried out in 30  $\mu$ l hybridization solution (90 mM NaCl, 83 mM guanidine thiocyanate, 8 mM  $MgCl_2$ , 17 nM EDTA, 0.02 % biotin, 0.1 % Tween-20, 70 mM mM Tris-HCl, pH 7.5,) with 218 nM biotinylated PNA oligomers. The arrays in the hybridization mix were incubated at 40°C for 60 min. Upon completion of hybridization, the arrays were washed with 50  $\mu$ l of 250 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween-20, at room temperature for 10 min. For detection of the biotinylated PNA oligomers that were hybridized on the composite particles of the invention, the arrays were incubated with Cy5.5-conjugated Streptavidin (18  $\mu$ g/ml) in 150 mM NaCl and 100 mM sodium phosphate, pH 7.2, at room temperature for 30 min. After washing with 15 mM NaCl, 10 mM Tris-HCl, pH 7.5, the arrays were examined by using a fluorescence microscope. Fluorescence emitted from the particles of the invention and the Cy5.5-labeled PNA oligomers were determined by using optical filters with specific wavelengths. The particles of the invention were decoded according to their color codes, and Cy5.5 fluorescence emitted from specific particles was evaluated by using a computer program (READ). Color-encoded particles devoid of magnetic character were used as controls in the assay. Results of the assay are shown in Figure 29 demonstrate that PNAs specifically hybridize to complimentary oligonucleotides coupled on the composite particles. Fluorescence signal intensity was determined from four types of the particles of the invention (I, II, III and IV) from two arrays (Chip A and B, for panels A and B, respectively) in the on-chip hybridization assay. The type I particles are the composite particles of the invention, whereas Types II, III and IV are three different types of the encoded particles of the invention. Types I and II are functionalized encoded particles with biotinylated oligonucleotides that are complementary to the PNA oligomers. Type III encoded particles are functionalized with an oligonucleotide with unrelated base sequence to the PNA. Type IV encoded particles have no oligonucleotides on their surface. Chip B serves as a negative control for chip A, which



was incubated with the hybridization mixture but without the target PNA. The symbol “n” denotes the number of particles on each type and the bars represent standard deviation of the mean.

## 5 Example 22. Immunoassay Using Fluorescent Magnetic Microparticles

In this Example, the composite particles of the invention are used for carrying-out immunoassays. The composite particles of the invention can be customized to display antibody of interest on their surface. An array of composite particles of the invention can be  
 10 exposed to the sample solution (such as serum solution) containing antigens of interest. Subsequent addition of fluorescently labeled secondary antibodies results in the formation of ternary fluorescent complex the concentration of which can be monitored by recording the fluorescence from the composite particle surface using methods disclosed in this invention. Arrays of the antibody functionalized composite particles of the invention can thus be used  
 15 for monitoring serum protein levels, and the binding pattern of the antigens of interest may be useful in protein profiling.

## Example 23: Alternative I: Magnetic Field-induced Array Formation

20 This Example illustrates the operation of Figure 22 and a sequence of reaction steps leading from the sequence-specific capture of mRNA molecules to the formation of a EFM bead-displayed cDNA array. Such an array will be useful in many applications. For example, in gene profiling, a planar cDNA array will be useful to profile mRNA concentrations; alternatively, the cDNA array can be probed directly using a pool of labeled DNA binding  
 25 agents or probes to profile the genes of interest within the array. The protocol, performed in a temperature controlled device, comprises the following steps: (1) introducing a set of the composite particle of the invention into a first compartment containing a pool of mRNA molecules, each particle type displaying a gene-specific oligonucleotide probe; mRNA molecules are permitted to anneal to their corresponding probes. Assay conditions for this  
 30 capture step are known in the art and are applicable herein; (2) perform on-particle reverse

transcription (RT) using particle-attached mRNA as template and release mRNA from the particles; (3) applying magnetic field from permanent magnet and wash while retaining the particles; (4) releasing the magnetic field and re-suspending the particles in buffer to perform the process of LEAPS; (5) direct the particles to a compartment containing  
 5 custom-designed chips and form a planar array of the composite particles of the invention using LEAPS.

#### **Example 24: Alternative II: Magnetic Field-induced Array Formation**

10 This Example illustrates the operation of Figure 22 and a sequence of reaction steps leading from the sequence-specific capture of mRNA molecules to the formation of a EFM particle-displayed cDNA array. The protocol set forth in Example 21 is used except that a planar array of the composite particles of the invention by applying a magnetic field to is formed using the application of a magnetic field and steps (4) and (5) of Example 21 are  
 15 omitted.

#### **Example 25: Multistep Assay Sequence Using Arrays of Encoded Magnetic Beads**

This Example illustrates a multi-step biochemical reaction protocol which integrates  
 20 the capture of genomic DNA fragments to a first set of magnetic nanoparticles, followed by magnetic separation and solid-phase amplification of captured “particle-tagged” fragments according to the general process described in Figure 22. This capture step is followed by a transformation step of concurrent “multiplex” PCR amplification reactions, each reaction containing a small set of primer pairs to produce solution-borne amplicons. These are pooled  
 25 and placed into contact with a set of the composite particles of the invention for multiplexed analysis invoking post-transformation mediated by application of a magnetic field.

The foregoing can be accomplished by the following steps :

#### **30 1. Sample Capture and First Transformation**

Given a solution containing fragments of genomic DNA, divide into four equal parts, aliquots are injected into four separate reaction compartments, equipped to permit temperature and control in accordance with standard PCR temperature cycling protocols. Into each compartment, one or more magnetic nanoparticles- tagged primers are injected. A particle-tagged primer is composed of magnetic nanoparticles displaying an oligonucleotide probe directed against a genomic DNA target fragment of interest, said probe also serving as a first primer of a subsequent polymerase-catalyzed primer extension reaction. The next step permits capture by hybridization of selected fragments to matching particle-displayed probes, said targets serving as templates in subsequent extension reaction. To each compartment, polymerase is added and one or more second primers as needed to permit template-directed extension of particle-coupled capture probes under temperature cycling concurrently in all compartments, thereby producing first extension products, anchored to the magnetic nanoparticles. A magnetic field is applied to form a planar array of magnetic nanoparticles in each compartment. The compartments are washed while retaining particle-- anchored extension products

A typical PCR protocol using particle-tagged primers to amplify a fragment of genomic DNA is as follows. An oligonucleotide probe corresponding to a specific target position within the fragment of interest is designed with variable 3' terminus aligned at or near the target position. Probes are synthesized to contain a 5' biotin-TEG with a 12 C spacer (Synthegen TX). Probes are attached to streptavidin-coated magnetic beads in accordance with standard reaction protocols such as the following: add to a magnetic bead suspension in 1X TE (100 mM Tris-HCl, 10 mM EDTA), 500 mM NaCl for 45 minutes at room temperature; wash beads with 1X TE, 150 mM of NaCl for 3X; and suspend in 50  $\mu$ l of same solution. Next, add 1  $\mu$ l of each bead suspension to to PCR mix containing 1X buffer (100 mM Tris-HCl, pH. 9.0, 1.5 mM Magnesium Chloride, 500 mM KCL), 40  $\mu$ M Cy5-labeled dCTP (Amersham Pharmacia Biotech NJ), and 80  $\mu$ M of the remaining three dNTPs, and 3 units of Taq DNA polymerase (Amersham Pharmacia Biotech NJ). Genomic DNA target fragments (40ng) are added to the PCR mix just prior to initiating extension. Under these conditions, ten cycles of amplification suffice to produce sufficient bead-

anchored extension product using a Perkin Elmer 9600 thermal cycler, a temperature cycle consisting of denaturation (30s at 90 C), annealing (30s at 55 C), and extension (20s at 72 C). Following completion of the extension reaction, beads typically are washed four times by centrifugation in 1X TE buffer.

5

## **2. Concurrent Instances of Second Amplification**

To each reaction compartment, polymerase was injected along with a set of one or more primer pairs designed to select regions within particle-anchored first extension products, each compartment receiving a unique set of such primer pairs. concurrent amplification of bead-anchored fragments under temperature cycling is allowed to occur, thereby producing, in each compartment, a set of specific amplicons as directed by the corresponding primer sets admitted into the compartment. Standard PCR reaction protocols (REF) apply to this step.

15

## **3. Pooling of Reaction Products, Post-Assay Array Assembly and Detection**

Solutions containing amplicons are combined to produce analyte solution and transfer to a detection compartment and placed into contact with a set of the encoded and magnetic nanoparticles of the invention, each particle displaying a sequence-specific oligonucleotide probe uniquely directed against one of the amplicons within the analyte solution. Annealing of amplicons to bead-displayed probes to form hybridization complexes is permitted to occur, and analysis of selected regions of target sequence by method of choice, e.g., differential thermal stability of hybridization complexes or probe elongation is performed. A magnetic field is applied to form planar array of the particles of the invention. An assay image signal is recorded. A decoding image is recorded to permit decoding of capture probe identities

Standard assay conditions for polymorphism analysis apply to this step, providing also conditions permitting formation of planar arrays of encoded functionalized

30

magnetic beads, such conditions being illustrated herein in connection with Example NN and Figure MM (SB: EFM bead array, induced by permanent magnet.)

#### **Example 26: Mixed Clusters and Arrays**

This Example illustrates an assay format wherein target DNA strands are attached to magnetic nanoparticles of the invention. Chemical attachment is readily accomplished using standard protocols of bioconjugate chemistry as known in the art. For example, strands of DNA are readily biotinylated and may then be attached to streptavidin-coated magnetic nanoparticles. Alternatively, particle-displayed DNA strands are produced by PCR using bead-tagged primers, as discussed in Example 21. The requisite set of encoded particles of the invention are produced by the methods provided herein. The particles are functionalized with specific oligonucleotide probes using standard methods of bio-conjugate chemistry as known in the art. In the course of the assay, hetero-structures composed of magnetic nanoparticles and encoded particles are formed as a result of the formation of a complex between binding partners, and these complexes are assembled into a planar array for detection.

Two alternatives are set forth herein. In the first alternative, the size of the optically encoded beads will typically significantly exceed that of the magnetic beads, a typical ratio of respective radii being 100:1; streptavidin-coated magnetic nanoparticles of 100 Å diameter are commercially available, as herein discussed in connection with Example 22. Next, a mixture of magnetic bead-displayed DNA target strands is combined with a set of color-encoded beads displaying oligonucleotide probes under conditions permitting hybridization of matching magnetic bead-tagged target targets to probes. Formation of hybridization complexes will “decorate” each color-encoded bead with a large number of magnetic nanoparticles to produce a magnetic shell, resulting in the formation of a particle of the structure and composition discussed herein.

Application of a magnetic field in accordance with the methods of the present invention, will then produce a planar array of the encoded and magnetic nanoparticles of the invention to

permit recording of a multicolor fluorescence image to identify captured DNA targets.

Alternatively, assay conditions may be chosen so as to favor the formation of clusters. That is, the magnetic nanoparticles of the invention acting as multi-dentate “ligands”, mediate “agglutination” of the encoded particles of the invention into a cluster of the encoded and magnetic of particles of the invention. Application of a strong magnetic field, typically producing magnetization in excess of 1000 Gauss, permits separation of these clusters from solution into a an expanded assembly of isolated clusters.

In a second alternative, the roles, and hence the relative sizes, of magnetic and encoded particles of the invention are reversed so that larger magnetic nanoparticles are decorated by the encoded particles. Application of a magnetic field in accordance with the conditions set forth in Example 19, (Dyna beads - solenoid) herein produces a planar array of magnetic nanoparticles wherein each particle will display an optical signal or not display an optical signal depending on whether the particle have been endowed with an optical identifier.

Both alternatives in this Example require reading of multi-optical signatures to identify captured targets. When particle optical loading encoding is achieved by application of a small number of encoding colors, typically two or three, and variation of the ratio of such encoding colors in accordance with standard methods as known in the art, multicolor imaging is readily

464-1045 US

achieved by standard multicolor fluorescence imaging using multiple filter sets as known in the art. Otherwise, if combinations of multiple distinct colors are used to produce the color code, then methods of multi-spectral imaging are generally available to record the assay image.